Investigation of the effects of Mechanical Strain in Human Tenocytes

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Abstract

Tendinopathies are a range of diseases characterised by pain and insidious degeneration. Although poorly understood, onset is often associated with physical activity. Metalloproteinases are regulated differentially in tendinopathy causing disruptions in extracellular matrix (ECM) homeostasis. An increase in the anti-inflammatory cytokine TGFβ has also been documented. This project aims to investigate the effect of cyclic tensile strain loading and TGFβ stimulation on protease and ECM protein expression by human tenocytes and begin to characterise the pathway of mechanotransduction.

Human tenocytes were seeded at $1.5x10^6$ cells/ml into collagen gels (rat tail type I, 1mg/ml) and stretched using a sinusoidal waveform of 0-5% at 1Hz using the Flexcell FX4000T™ system. Cultures were treated with or without 1ng/ml TGFβ1 or inhibitors of TGFβRI, metalloproteinases, RGD, Mannose-6-phosphate, integrin β1 and a thrombospondin as appropriate. qRT-PCR and a cell based luciferase assay were used to assess RNA and TGFβ activity respectively.

The prolonged application of 5% cyclic mechanical strain in a 3D culture system induced an anabolic response in protease and matrix genes. In most genes changes in gene expression with loading was mirrored with TGFβ stimulation. We also demonstrated that the inhibition of the TGFβRI abrogated the strain induced changes in mRNA. TGFβ activity was increased with 48 hours mechanical strain although there was no increase in mRNA or total TGFβ. This indicates that TGFβ activation plays an important role in the mechanoregulation of gene expression. Inhibition of potential mechanisms of TGFβ activation including; serine protease or metalloproteinase activity, integrin or thrombospondin interaction with latent TGFβ showed no effect upon TGFβ activation or gene regulation with strain. Therefore strain mediated TGFβ activation may occur via a novel mechanism. By understanding mechanotransduction, we may be able to determine whether dysregulation of this system is involved in the development of tendinopathy.

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CHAPTER 1: Introduction

Tendinopathies are a range of diseases that are a significant cause of morbidity and represent a sizable proportion of referrals to general practitioners (Badley and Tennant 1993; McCormick, Charlton et al. 1995). Although these individuals do not show risk of mortality, they do suffer with a decrease in quality of life, involving pain and decreased mobility (Khan, Cook et al. 2000). In addition, long periods of rehabilitation are often involved (Clegg, Strassburg et al. 2007). Characteristic symptoms include; chronic pain, swelling, localised tenderness, impaired movement, rupture or microinjury (through overuse) and insidious degeneration of the tendon (Paavola, Kannus et al. 2002). Vascular profusion, impingement, trauma and repetitive unequally distributed strain have been implicated in the development of tendinopathy (Riley 2004). Relatively little is understood about the underlying mechanisms, though onset is often associated with increased age and physical activity (Badley and Tennant 1993; Paavola, Kannus et al. 2002; Fedorczyk 2006; Dudhia, Scott et al. 2007).

Tendons predominantly affected are those exposed to higher levels of mechanical strain such as the supraspinatus, Achilles, patella and the lateral and medial epicondyle tendon (Woo, Renström et al. 2007). Although prevalent in the general population, athletes are the most commonly affected; for example, there is a high incidence of Achilles tendinopathy in the running population (Paavola, Kannus et al. 2002; Woo, Renström et al. 2007). Mechanical strain is therefore considered to be one of the major contributing factors to the onset of the disease. However, due to our inadequate understanding of the disease pathology, treatment is restricted to pain relief, exercise, cryotherapy, non-steroidal ant-inflammatory drugs and surgery (Khan, Cook et al. 2000; Paavola, Kannus et al. 2002). However, these treatments are often ineffective (Almekinders and Temple 1998; Khan, Cook et al. 2000; Paavola, Kannus et al. 2002), even after corrective surgery only 60-85% are able to return to sporting exercise (Woo, Renström et al. 2007). In order to develop effective treatments we must first understand the disease mechanistically.

1.1. TENDON STRUCTURE

Tendon is characterized as dense fibrous connective tissue (Riley 2004). Tendon is well known for its hierarchal structure (Khan, Cook et al. 1999). Tightly packed collagen fibrils make up fibres, sub fascicles, fascicles and tertiary fibre bundles and are enclosed in the endotenon giving tendon its strength and flexibility (Figure 1.1) (Kastelic, Galeski et al. 1978). Tenocytes form an elaborate interconnecting network of cell processes which run longitudinally within the fibre bundles as well as laterally (so that each fibre bundle is enclosed in sheet like cell processes). Tenocytes in this arrangement are thought to communicate through GAP junctions (McNeilly, Banes et al. 1996). At the periphery of the tendon are layers of flattened tenocytes (2-3) that form what is known as the epitenon (Kastelic, Galeski et al. 1978). Tenocytes of the epitenon inner layers communicate through GAP junctions with the cells of the outer tendon proper (McNeilly, Banes et al. 1996). Tendon is made up of a complex array of collagens, proteoglycans and glycoproteins, some of which are poorly characterised; here we describe tendon composition further.

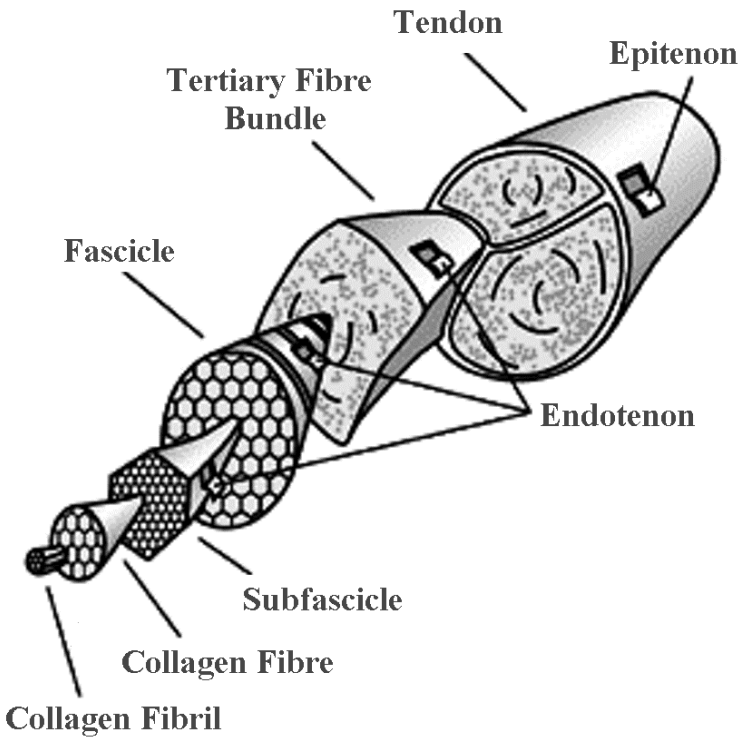

Figure 1.1. Tendon Hierarchal structure.

The tendon is made up of a complex hierarchal structure. Individual collagen fibrils group to form collagen fibres, multiple fibres form subfascicles. Subfascicles group to form the tertiary fibre bundles. Finally Tertiary fibre bundles group to form the tendon. Subfascicles, fascicles and tertiary fibre bundles are all surrounded in the endotenon. The entire tendon is enclosed in the epitenon. Adapted from (Riley 2005)

1.1.1. Collagens of tendon

Twenty eight different types of collagen have been characterised to date (Kadler, Baldock et al. 2007). Collagens are generally composed of a right handed triple helix made up of homo or heterotrimers of polypeptide chains known as α chains. There are 42 different α chains that are combined in triplicate to create different types of collagen (Table 1.1). Each α chain within the triple helix is made up of a left-handed helix with an expanse of 18 amino acids per turn of the helix. Each α chain contains a glycine repeat every three amino acids to form Gly-X-Y, where X and Y positions are often occupied by proline and hydroxyproline respectively. Specific residues (proline and lysine residues) are modified by posttranslational enzymatic hydroxylation. Hydroxyproline content is important for the formation of hydrogen bonds within the helix (Gelse, Poschl et al. 2003; Riley 2005; Kadler, Baldock et al. 2007).

Collagen is synthesised as soluble procollagen, flanked with a C-terminal trimeric globular propeptide domain and an N-terminal trimeric propeptide domain (Figure 1.2). The C-terminal globular domain is important in the assembly of the trimeric collagen monomers (Gelse, Poschl et al. 2003). Upon secretion the N-terminal and C-terminal pro regions are cleaved to produce mature collagen. This is mediated by procollagen metalloproteinases such as ADAMTS 2, 3 and 14 which cleave N-terminal procollagen (Colige, Beschin et al. 1995; Colige, Li et al. 1997; Fernandes, Hirohata et al. 2001; Wang, Lee et al. 2003) and BMP-1 which cleaves the C-terminal of procollagen (Li, Sieron et al. 1996). Mature collagen spontaneously assembles to form string like aggregates known as fibrils. Although this occurs spontaneously, a range of extracellular matrix components are involved in the temporal and spatial regulation of collagen fibrillogenesis (Kadler, Hill et al. 2008). Collagens form different structural conformations and they are grouped accordingly; fibril forming collagen, meshwork forming collagen, beaded filament collagen and fibril-associated collagen with interrupted triple helix (FACIT) (Riley 2005). For a more detailed review of basic collagen structure and fibril formation see the following articles (Kadler, Holmes et al. 1996; Ottani, Martini et al. 2002; Gelse, Poschl et al. 2003; Canty and Kadler 2005; Riley 2005; Kadler, Hill et al. 2008).

The main constituent of the tendon hierarchal structure is type I collagen, it forms ~95% of tendon mid-substance (Rees, Flannery et al. 2000). Type I collagen is a fibrillar collagen

made up of a heterotrimer by two identical α1(I) chains and one α2(I) chain. Triple helices align with the longitudinal axis of loading (Screen, Shelton et al. 2005). Type I collagen is therefore important in longitudinal strength (Riley, Harrall et al. 1994).

Type III collagen is made up of a homotrimer of α1(III) chains. Type III collagen is more compliant in physiology, forms a meshwork of thinner fibres than type I collagen and constitutes approximately 2% of tendon. It is also a fibrillar collagen predominantly localized to the surrounding areas of tendon fibres, endotenon and epitenon (Duance, Restall et al. 1977; Riley, Harrall et al. 1994).

Other types of collagen are also present, although less abundantly, such as fibril forming collagens type II and type V, network forming collagens such as type IV, type VI filamentous collagen, and types XII and XIV which are fibril-associated (Table 1) (Riley, Harrall et al. 1994; Riley 2004; Riley 2005). Collagen was first thought to be a static protein of little interest, however it is now known that collagen is involved in constant turnover, which enables ECM to adapt to the environment (Laurent 1987).

Collagen	Structure/Type	Location and function
Type I	Fibril-forming $[\alpha1(I)]_2$, α2(I)	Main constituent of tendon (~95% of total collagen)
Type II	Fibril-forming $[\alpha1(II)]_3$	Restricted to fibrocartilage of the tendon forms less-organized meshwork
Type III	Fibril-forming $[\alpha1(III)]_3$	Normally restricted to endotenon and epitenon forms smaller, less-organized fibrils
Type IV	Forms meshwork $[\alpha1(III)]_2$, α1-6(III)	Basement membrane of blood vessels
Type V	Fibril-forming α1(V), α2(V), α3(V)	Core of type I collagen fibril; forms template for fibrillogenesis
Type VI	Beaded filaments α1(VI), α2(VI), α3(VI)	Cell-associated found in 'seams' between fibrils
Type IX	FACIT α1(IX), α2(IX), α3(IX)	Mediates cell–matrix interactions with type II collagen fibril surface
Type X	Forms meshwork $[\alpha1(X)]_3$	Restricted to insertion fibrocartilage May be associated with mineralisation
Type XI	Fibril-forming α1(XI), α2(XI), α3(XI)	Core of type II collagen fibril forms template for fibrillogenesis
Type XII	FACIT $[\alpha1(XII)]_3$	Mediates cell–matrix interactions with type I collagen fibril surface
Type XIV	FACIT $[\alpha1(XIV)]_3$	Mediates cell–matrix interactions with type I collagen fibril surface

Table 1.1. Collagen composition of tendon ECM

[Adapted from (Gelse, Poschl et al. 2003; Riley 2005)] Abbreviations: FACIT, fibril-associated collagen with interrupted triple helix

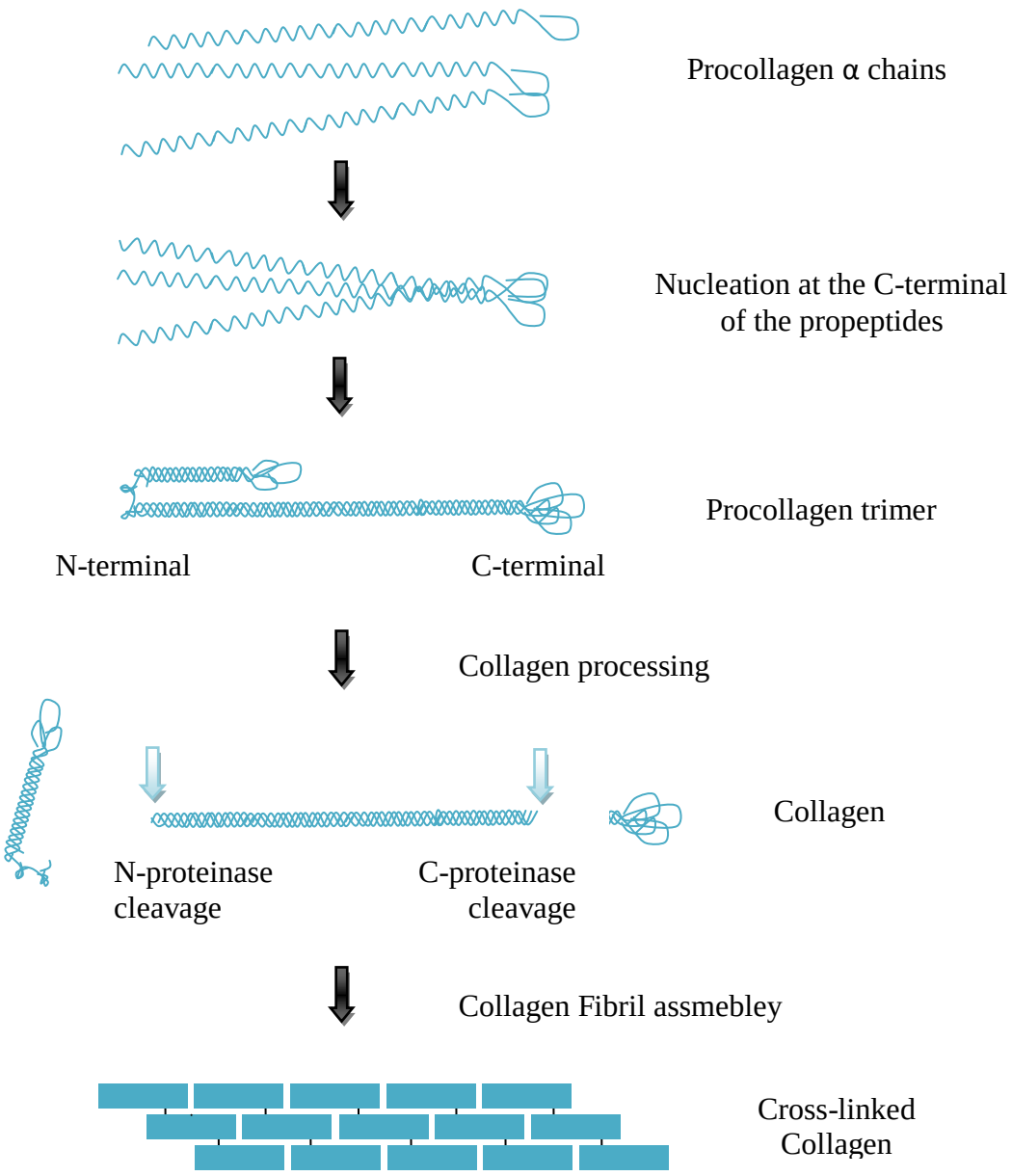

Figure 1.2. Collagen fibril formation. Procollagen α chains are synthesised in the endoplasmic reticulum. The collagen α chains aggregate to form a trimer, this interaction begins at the C-terminal propeptide. Procollagen form a rod like triple helical domain flanked by N and C-terminal globular propeptides. Collagen propeptides are cleaved at the N and C terminal by metalloproteinases to form a mature collagen molecule. Mature collagen molecules spontaneously aggregate to form fibrils. Crosslinking of collagen by lysyl oxidase stabilises the complex. Adapted from (Kadler, Holmes et al. 1996; Gelse, Poschl et al. 2003; Canty and Kadler 2005)

1.1.2. Proteoglycans of tendon

Tendons are composed of 1% proteoglycan (Vogel and Heinegard 1985; Riley 2005), the most abundant non-collagenous molecules in tendon (Screen, Shelton et al. 2005). The most plentiful of these include small leucine rich proteoglycans (SLRP) such as decorin and biglycan and large aggregating proteoglycans such as aggrecan (Rees, Flannery et al. 2000) (See summary of tendon proteoglycans in Table 1.2). Proteoglycans form bridges between collagen fibrils that increase tendon strength and reduce the sliding of fibrils (Screen, Shelton et al. 2005). Sulphated Glycosaminoglycans (GAG) such as keratan sulphate, dermatan sulphate and chondroitin sulphate, can form part of Proteoglycan molecules (Rees, Flannery et al. 2000). Tendons have different total GAG proportions according to function, for example the supraspinatus has a high GAG level compared to tendons under less strain (Riley, Harrall et al. 1994). Not only do proteoglycans function as structural features of tendon but they are also involved in a range of important biological processes (Hardingham and Fosang 1992). Proteoglycan and GAG complexes are important in tendon resistance to load: for example aggrecan has many GAG regions which have a very high negative charge (sulphate and carboxylate groups), this attracts counter ions causing the osmotic potential to increase and therefore the tissue retains water. Water saturation is resisted by the collagen network, enabling the tendon to maintain a turgid structure (Hardingham and Fosang 1992). Areas containing high levels of proteoglycan are able to withstand elevated compressive loading, this is evident in tendons which undergo high level compressive loading such as the supraspinatus in the rotator cuff (Riley 2005).

Small leucine rich proteoglycans (SLRP)

SLRPs present in tendon include decorin, biglycan, fibromodulin and lumican, although decorin and biglycan are the most abundant. They contain a small core protein (~40kDa) consisting of a central domain containing leucine rich repeats involved in collagen binding and an N-terminal region capable of binding GAG (Figure 1.3) (Hardingham and Fosang 1992). Fibromodulin and lumican consist of a core protein bound to GAG side chains; fibromodulin contains up to 100 keratan sulphate chains and both fibromodulin and lumican bind up to 21 chondroitin sulphate chains (Funderburgh, Caterson et al. 1987; Oldberg, Antonsson et al. 1989). Decorin and biglycan contain a similar protein core and are able to bind chondroitin sulphate as well as dermatan sulphate, although binding of one

dermatan sulphate to decorin and two chondroitin sulphate to biglycan is more common in tendon ECM (Hardingham and Fosang 1992).

Decorin, biglycan, fibromodulin and lumican have the ability to bind fibrillar collagen types I and II and are involved in the regulation of collagen fibrillogenesis through the interaction of their core protein. These proteoglycans reduce the thickness of collagen fibres and delay the process of fibril formation (Danielson, Baribault et al. 1997; Chakravarti, Magnuson et al. 1998; Svensson, Aszodi et al. 1999). Biglycan can also bind the N-terminal of type VI collagen with high affinity via its core protein and GAG chains. Through this interaction, biglycan is involved in the assembly of type VI collagen into a hexagonal network, where GAG chains play a key role by separating the collagen molecules while the assembly is initiated (Wiberg, Heinegard et al. 2002).

Decorin, biglycan and fibromodulin are able to bind TGFβ via conserved regions of their core protein, however fibromodulin appears to bind with greater affinity to TGFβ1 and TGFβ2 isoforms compared to the other proteoglycans, this is due to the inhibition of this interaction by GAG chains i.e. chondroitin sulphate. On the other hand, fibromodulin was less effective at binding TGFβ3 than decorin and biglycan (Hildebrand, Romaris et al. 1994). This interaction may lead to the sequestration of TGFβ in the ECM and therefore regulate the availability of TGFβ (for more details of TGFβ release and activation see section 1.9). Decorin is also able to inhibit the activity of TGFβ, and as TGFβ can induce decorin, the inhibition of TGFβ activity by decorin may act as a negative feedback loop (Hardingham and Fosang 1992). SLRPs are therefore key regulators in collagen fibrillogenesis and the spatial and temporal regulation of TGFβ.

Large Proteoglycans

Aggrecan is the most highly characterised of the proteoglycans. It is a large proteoglycan with a core protein of approximately 230-250kDa; it is most highly expressed in areas of articular cartilage where the need to withstand compressive load is at its highest (Hardingham and Fosang 1992). In tendon aggrecan is predominantly expressed in areas exposed to high level compressive load. As I have previously mentioned, resistance to compressive load involves the interaction of water and aggrecan via its many GAG chains. Aggrecan contains a large number of keratan sulphate and chondroitin sulphate chains (see Figure 1.3 for aggrecan structure), in fact carbohydrate constitutes 90% of aggrecan (Hardingham and Fosang 1992). The GAG binding domain contains 3 distinct sites, one

region which binds to keratan sulphate and two regions that bind to chondroitin sulphate (often termed CS1 and CS2). As well as the GAG binding domain, aggrecan also contains 3 globular domains (known as G1, G2 and G3), G1 and G2 are located at the N terminus and G3 is located at the C terminus with the GAG binding domain between the G2 and G3 domains (Riley 2005). The G1 domain containing an immunoglobulin domain may be involved in cell recognition. The G2 domain contains a proteoglycan tandem repeat may be involved in binding of hyaluronan. The G3 domain may be involved in the intracellular processing and trafficking of aggrecan as well as the targeted binding of specific GAG chains (Hardingham and Fosang 1992).

Versican is also a large proteoglycan with a very similar structure to aggrecan, although it lacks the G2 domain and carries far less chondroitin sulphate (from 0-23 chains depending on the splice variant) and no keratan sulphate (see figure 1.3 for versican structure). Versican is expressed as four splice variants generated from one gene. The GAG binding domain of versican is divided into sections known as GAGα (exon 7) and GAGβ (exon 8), and the four spice variants of versican are created by alternate splicing of these domains (Wight 2002). The C terminal G3 domain of both versican and aggrecan contains an epidermal growth factor-like domain, a C-type lectin-like domain and a complementary regulatory protein-like domain (Hardingham and Fosang 1992). Versican is involved in cell adhesion in the pericellular matrix which in turn can modulate cell proliferation, migration and phenotype, i.e. increased expression of versican in the pericellular matrix can induce a change from fibroblastic to myofibroblastic phenotype (Hattori, Carrino et al. 2011).

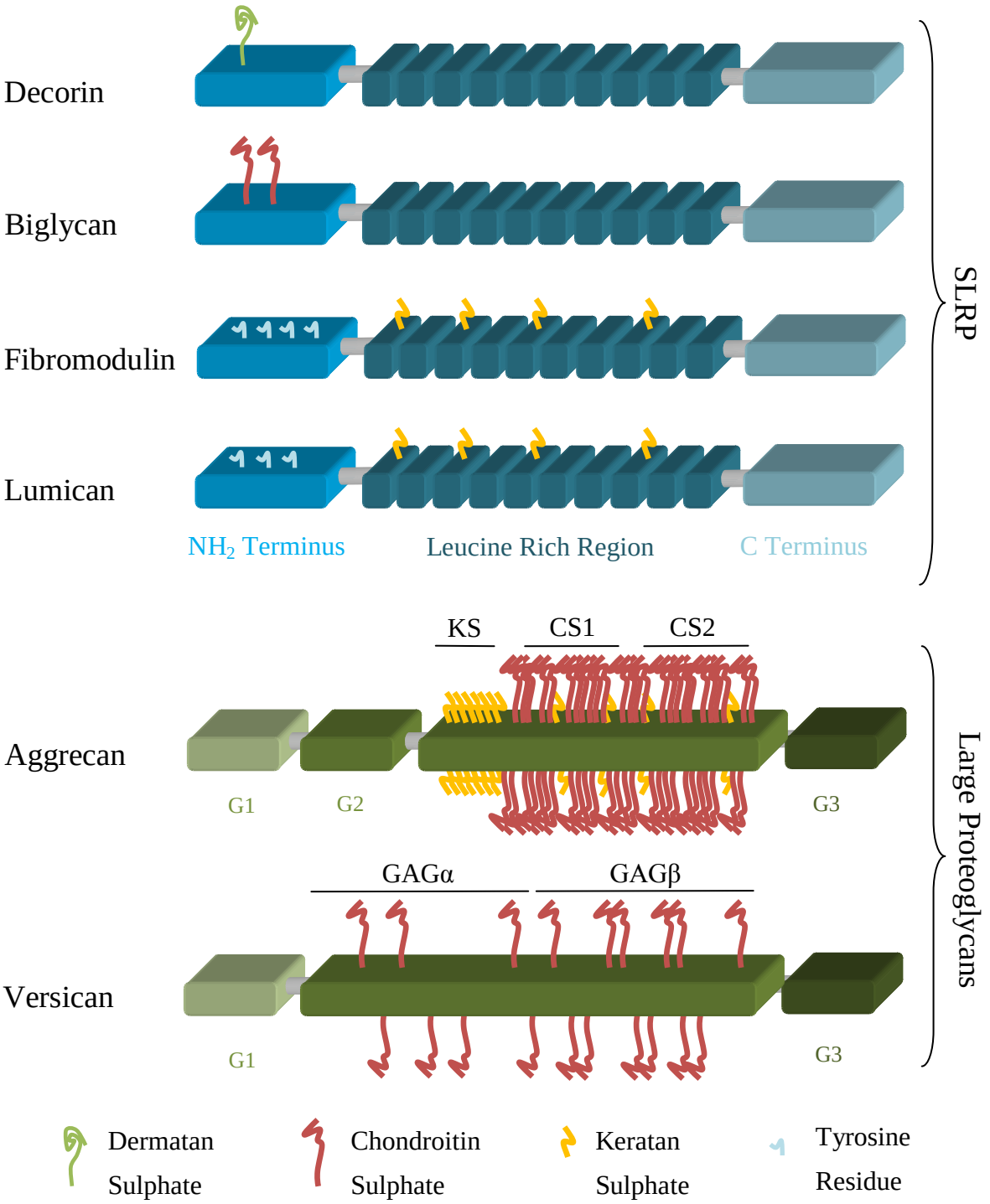

Figure 1.3. Proteoglycan Structure in tendon. Decorin, biglycan, fibromodulin and lumican are small leucine rich proteoglycans (SLRP) - they have a similar core protein structure and bind different glycosaminoglycans (GAG) (keratan sulphate, chondroitin sulphate or dermatan sulphate) at either the NH2 terminus or the leucine rich region. Aggrecan and versican are large proteoglycans also known as hyalectans. Aggrecan contains G1, G2 and G3 domains whereas versican contains only the G1 and G3 domains. Aggrecan binds to keratan sulphate at one domain (KS) and chondroitin sulphate at two domains (CS1 and CS2). Versican bind only chondroitin sulphate. Versican is expressed as four isoforms, which are made up of spice variants of the α and β GAG binding domain. Adapted from (Hardingham and Fosang 1992; Riley 2004; Riley 2005).

1.1.3. Glycoproteins of tendon

The main non-collagen glycoproteins present in the tendon include elastin, tenascin-C, fibrillin, fibronectin, laminin, link protein, thrombospondin and COMP (See summary of tendon glycoproteins in Table 1.2). Elastin is secreted as tropoelastin which is unstable and soluble. However once elastin is fully formed into fibres it is characteristically stable and insoluble, with many hydrophobic amino acids and a high proportion of glycine and proline (Uitto 1979). Elastin fibres are made up of polypeptide chains connected via highly stable desmosine and isodesmosine crosslinks which are essential for strength and elasticity (Uitto 1979). Elastin is the main component of elastic fibres and although less than 2% of the tendon dry weight is made up of elastin, it is has important elastic properties including the preservation of the crimp (Kastelic, Galeski et al. 1978; Riley 2005). Elastin expression is highest at the insertion where the tendon is exposed to the highest tensional forces and lowest in areas of fibrocartilage (Ritty, Ditsios et al. 2002).

Fibrillin is the main constituent of extracellular microfibrils. They are cysteine rich glycoproteins consisting of numerous calcium binding epidermal growth factor domains. They polymerise extracellularly in a head to tail formation to form parallel bundles which are stabilised by calcium and the action of transglutaminase (Ramirez and Pereira 1999). Fibrillins are commonly associated with elastic fibres as well as microfibril associated glycoproteins 1 and 2 (MAGP1 and MAGP2) (Ritty, Ditsios et al. 2002). Fibrillins form a template for tropoelastin in elastic fibrillogenesis (formation of elastin fibres). This is mainly achieved by fibrillin-2. Fibrillin-1 is involved in the resistance to tensional force (Ramirez and Pereira 1999). Fibrillin also plays a key role in the sequestration of latent TGFβ in the ECM (for more details see section 1.9) (Ramirez and Rifkin 2009) and interacts with cells via $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Bax, Bernard et al. 2003).

Thrombospondin-1 (TSP1), Fibronectin, COMP and tenascin-C are involved in cell-matrix interactions (Riley 2004). TSP1 is a large heterotrimeric and adhesive glycoprotein. Each of its three components has a molecular weight of 140kDa and contains three repeating subunits (I, II and III) (Wolf, Eddy et al. 1990). TSP1 is able to bind its receptor, CD36, on the cell surface (Yehualaeshet, O'Connor et al. 1999) and also other components of the ECM such as collagen and laminin (Wolf, Eddy et al. 1990). It is involved in the activation of transforming growth factor β, inducing a conformational change in the latent TGFβ

complex upon binding via an LSKL motif (Schultz-Cherry and Murphy-Ullrich 1993). This can also occur while TSP1 is bound to its receptor (Yehualaeshet, O'Connor et al. 1999). TSP1 is also involved in angiogenisis. TSP1 contains two domains which have anti-angiogenic properties, each can induce anti-angiogenic effects independantly (Tolsma, Volpert et al. 1993).

Fibronectin is made up of either one or two ~250kDa subunits linked by a disulphide bond. Different isoforms of fibronectin are generated by alternative splicing of a single gene. Fibronectins are implicated in a wide range of processes including cell adhesion, growth and differentiation, mainly involving the interaction of cells with the ECM (Pankov and Yamada 2002). Fibronectin can bind heparin, collagen and fibrin as well as facilitate cellular interactions through integrins. A wide range of integrins, for instance $\alpha_5\beta_{1,}$ can bind to fibronectin via an RGD binding motif and a non RGD N-terminal region which may facilitate intracellular signalling (Pankov and Yamada 2002). Although fibronectin is expressed at relatively low levels in normal tendon it is increased in both chronic and ruptured tendinopathies (Ireland, Harrall et al. 2001; Jelinsky, Rodeo et al. 2011).

Cartilage oligomeric matrix protein (COMP), also known as thrombospondin-5, is a large 524kDa protein made up of 5 disulphide linked subunits each with EGF-like and calcium binding thrombospondin-like domains (Oldberg, Antonsson et al. 1992). Initial studies showed that COMP was restricted to cartilage (Hedbom, Antonsson et al. 1992). However more recent studies have confirmed that COMP makes up 3% of the tendon dry weight (DiCesare, Hauser et al. 1994). It is more abundant in adult tendon compared to foetal tendon; it varies according to the site of tendon and is higher in tendons exposed to higher tensional force (DiCesare, Hauser et al. 1994; Smith, Zunino et al. 1997). COMP is also implicated in collagen fibrillogenesis (Halasz, Kassner et al. 2007).

Tenascin-C is a large glycoprotein, a smaller (200kDa) and a larger (300kDa) isoform are produced through alternative splicing of a single transcript, which are expressed in normal and degenerate tendon respectively (Riley, Harrall et al. 1996). Tenascin-C is absent in areas of poorly organised matrix but highly expressed in areas of compressive loading (Riley, Harrall et al. 1996; Martin, Mehr et al. 2003). Binding of tenascin-C to fibronectin prevents cellular interaction with the ECM, the lack of matrix interaction causes the cells to become more rounded, i.e. more chondrocyte like. This change in morphology functions to protect the cells from excessive compressive loads (Martin, Mehr et al. 2003). Tenascin-

C may also be involved in the organisation of collagen fibrils in normal tendon (Riley, Harrall et al. 1996).

Other less abundant glycoproteins present in tendon include laminin, link protein and fibulin. Laminin is a major component of the basement membrane (Timpl, Rohde et al. 1979). Link protein is a 45kDa globular protein of which there are 3 isoforms (Link protein 1-3). Link proteins bind to large proteoglycans such as aggrecan and helps to stabilise their interaction with hyaluronan (Neame, Christner et al. 1986). Fibulin is expressed as six different genes (Fibulin1-6); Fibulin-1 for example has 4 isoforms termed Fibulin-1 A-D which are splice variants of a single gene (Tran, Mattei et al. 1997). Fibulin is reported to bind to various matrix components, including elastin (Sasaki, Gohring et al. 1999), and is involved in matrix stabilisation (Olin, Morgelin et al. 2001). Fibulin can bind aggrecan as well as ADAMTS1; this co-localisation is thought to increase aggrecan proteolysis via ADAMTS activity (Lee, Rodriguez-Manzaneque et al. 2005).

Proteoglycan	Type	Location and function
Decorin	SLRP	Binds collagen, affects collagen-fibril formation Binds growth factors
Biglycan	SLRP	Binds collagen, affects collagen-fibril formation Binds growth factors
Fibromodulin	SLRP	Binds collagen, affects collagen-fibril formation Binds growth factors
Lumican	SLRP	Binds collagen, affects collagen-fibril formation
Aggrecan	Hyalectan	Resists compression; most prominent in fibrocartilage
Versican	Hyalectan	Lubricates boundary between adjacent fibrils?
Glycoprotein		
Elastin	Branched network	Forms elastic fibres; provides elastic properties of tissue
Fibrillin	Linear arrays	Forms elastic fibres; provides elastic properties of tissue, binds LTBP
Tenascin-C	Branched molecule	Mediates cell–matrix interactions; forms 'seams' with versican
COMP	Branched molecule	Mediates cell–matrix interactions; role in fibril formation
Fibronectin	Modular protein	Mediates cell–matrix interactions; role in tendon healing
Laminin	Modular protein	Component of basement membranes
Link protein	Globular protein	Stabilises proteoglycan–hyaluronan interactions
Thrombospondin	Modular protein	Mediates cell–matrix interactions
Glycosaminoglycans		
Keratan Sulphate	Linear polymer	Resists compression; Binds Fibromodulin and Aggrecan in tendon
Dermatan Sulphate	Linear polymer	Resists compression; Binds decorin in tendon
Chondroitin sulphate	Linear polymer	Resists compression; Binds biglycan, aggrecan and versican in tendon

Table 1.2. Non-collagen composition of tendon ECM

Adapted from (Riley 2005) Abbreviations: COMP, cartilage oligomeric matrix protein; SLRP, small leucine-rich repeat proteoglycan; LTBP, Large TGFβ binding protein.

1.2. TENDON CELL POPULATION

Tendon fibroblasts or tenocytes represent the major cell population within the tendon; they are relatively sparsely distributed throughout the tissue, although some areas are more populated than others (Clegg, Strassburg et al. 2007). Tenocytes have elongated nuclei and are located in longitudinal alignment between the collagen fibres (Clegg, Strassburg et al. 2007). Cellular protrusions (both sheet like [lateral and longitudinal] and long/thin [longitudinal]) are thought to connect adjacent tenocytes though GAP junctions in multiple directions throughout the tendon (Senga, Kobayashi et al. 1995; McNeilly, Banes et al. 1996; Clegg, Strassburg et al. 2007). Tenocytes have also been characterized by their close association with local collagen fibrils (McNeilly, Banes et al. 1996). Tenocytes are involved in the deposition of matrix components and also the expression of matrix degrading proteases involved in the turnover of the ECM. Other cell populations are also present but are significantly less abundant; these include; mesenchymal stem cells, synovial cells, fibrochondrocytes, endothelial cells, nerve cells and smooth muscle cells (Salingcarnboriboon, Yoshitake et al. 2003; Riley 2005).

1.2.1. Tendon cell markers

A number of genes have been selected as possible markers of tendon cell phenotype; these include: Scleraxis, Tenomodulin, Tenascin-C, COL1A1 and decorin (Salingcarnboriboon, Yoshitake et al. 2003; Taylor, Vaughan-Thomas et al. 2009). Scleraxis is a basic helix-loop-helix transcription factor involved in the regulation of collagen expression (Lejard, Brideau et al. 2007; Espira, Lamoureux et al. 2009). Scleraxis has been determined as a marker for tendon and ligament progenitor cells during development (Schweitzer, Chyung et al. 2001). It was reported that Scleraxis expression was significantly different between bone cells and tendon cells, however, it was not significantly different between tendon and cartilage (Taylor, Vaughan-Thomas et al. 2009). In addition, a genome wide array study of both rat and human adult tissue showed that Scleraxis is not only expressed in tendon but also in the muscle midsubstance (Jelinsky, Archambault et al. 2010). This suggests that Scleraxis is not a definitive marker of tendon phenotype.

Tenomodulin is a type II transmembrane glycoprotein expressed in 3 isoforms produced from splice variants of a single gene. Tenomodulin is involved in the regulation of cell proliferation (Docheva, Hunziker et al. 2005; Qi, Dmochowski et al. 2012) and thought to

be a marker of tenocytes in the later stages of tendon formation (Shukunami, Takimoto et al. 2006). A genome wide study showed that tenomodulin was consistently expressed in rat and human adult tendon, with significantly lower expression in a wide range of other tissues (Jelinsky, Archambault et al. 2010). Therefore tenomodulin may be a good candidate as a marker of adult tendon phenotype. However, in an equine study tenomodulin expression was not significantly different between tendon and bone derived cells (Taylor, Vaughan-Thomas et al. 2009).

Tenascin-C, COL1A1 and decorin are components of the tendon ECM (Hardingham and Fosang 1992; Riley, Harrall et al. 1996; Rees, Flannery et al. 2000). In an equine study of tendon, cartilage and bone, COL1A2 gene expression was no different in tendon than bone cells. COMP and decorin were increased in tendon compared to bone cells, but was not significantly different from cartilage. Also tenascin-C was significantly lower in tendon than in bone cells; however a decrease in expression is not characteristic of an ideal cell marker (Taylor, Vaughan-Thomas et al. 2009). Therefore, not one of these genes alone can distinguish between tendon cells from either cartilage or bone derived cells. It has therefore been proposed that multiple genes should be used to discriminate between fibroblast cells types (Taylor, Vaughan-Thomas et al. 2009). This indicates that there are no definitive tendon 'markers' that can be used to distinguish between tenocytes and other cell types (Riley 2005; Riddle, Taylor et al. 2006).

A genome wide study of gene expression from a wide range of adult human and rat tissue showed that Thrombospondin 4 was more highly expressed in tendon than any other tissue; including muscle, cartilage and bone (Jelinsky, Archambault et al. 2010). The function of thrombospondin-4 is unknown; however, it is a large glycoprotein capable of binding to collagen and COMP (Narouz-Ott, Maurer et al. 2000; Sodersten, Ekman et al. 2006). Although it was not possible to differentiate between adult tendon and ligament cells by comparing their expression of thrombospondin 4 (Jelinsky, Archambault et al. 2010), this indicates that thrombospondin 4 is a potential marker of tendon/ligament phenotype.

1.3. VASCULARISATION

The vascularisation of tendon is relatively low compared to other tissues (Schmidt-Rohlfing, Graf et al. 1992; Khan, Cook et al. 1999) and this is thought to reduce healing capabilities of the tendon once damage has occurred (Ahmed, Lagopoulos et al. 1998). Tendon cells are also nourished by the diffusion of synovial fluid in the absence of a more vascularised network (Gelberman 1985). Some tendons have regions of further reduced vascularity, these include the supraspinatus (Rathbun and Macnab 1970; Determe, Rongieres et al. 1996), posterior tibialis (Frey, Shereff et al. 1990) and Achilles (Schmidt-Rohlfing, Graf et al. 1992). Areas of avascularity are localised to areas of the tendon that are exposed to compressive load (Rathbun and Macnab 1970), this suggests that differences in mechanical loading are involved in the regulation of vascularity. Poorly vascularised tendons appear to be those most commonly affected by tendinopathy and areas of tendon rupture also show signs of avascularity (Rathbun and Macnab 1970). Conversely, in degenerative tendon areas of increased vascularisation have been reported (Rathbun and Macnab 1970). However the increase in vascularity seen in degenerate tendinopathy is thought to be a response rather than a cause of the disorder (Fenwick, Hazleman et al. 2002). This increase in vascularity can be explained by the following scenario; The lack of an oxygen supply to the tendon as a result of avascularity may induce hypoxia, hypoxia induces the expression of vascular endothelial growth factor (VEGF) which in turn increases vascularisation (Fenwick, Hazleman et al. 2002). Decreased vascularity is therefore thought to be a factor in the development of tendinopathy, however how this occurs is not fully understood (Rathbun and Macnab 1970; Frey, Shereff et al. 1990; Determe, Rongieres et al. 1996; Fenwick, Hazleman et al. 2002).

1.4. TENDON FUNCTION

Tendon primarily functions as a force transmitter between muscle (myotendinous junction) and bone (enthesis) (Rees, Flannery et al. 2000; Screen, Chhaya et al. 2006), in addition to joint stabilization, stress absorption and muscle damage restriction (Clegg, Strassburg et al. 2007). The Achilles tendon is subjected to high levels of strain in comparison to other tendons (Wall and Banes 2005) and storage and release of mechanical strain, through elastic recoil, plays an important role (Alexander 2002). Tendon hierarchal structure enables the tendon to dissipate a certain level of load through fibre alignment and muscle rotation as well as straightening of the crimp (Riley 2004; Screen, Lee et al. 2004). This is illustrated by the toe region of the stress-strain curve (Figure 1.4) where there is a non-linear response to an increase in mechanical strain. In addition, the extension of tropocollagen molecules facilitated by the rotation of the triple helix (Puxkandl, Zizak et al. 2002) as well as collagen molecule sliding contributes to fibril elongation (Purslow, Wess et al. 1998). Therefore tenocytes themselves are not exposed to the same mechanical strain as the whole tendon fascicle (Screen, Lee et al. 2004). The level of strain transmitted to tenocytes in vivo is approximately 6% (Screen, Lee et al. 2004).

The ‘crimp’ refers to the concertina type morphology of relaxed collagen fibres, the possible role of crimp is to absorb a level of tension via the straightening out of fibres under longitudinal strain (Kastelic, Galeski et al. 1978). The stress-strain curve illustrates the response of the tendon to stress (load per area) through changes in strain (percentage elongation) (Figure 1.4). The toe region of the stress-strain curve demonstrates the effect of crimp where cellular deformation is limited. With increased stress the collagen fibres are straightened. The amount of cellular deformation increases linearly, if the tendon is exposed to enough strain, until the tendon fibrils and other higher components of the tendon hierarchy rupture. Physiologically relevant strain levels perceived by the cell are thought to reach approximately 6% (Lavagnino, Arnoczky et al. 2003; Screen, Lee et al. 2004). Finite element model studies have shown that the cells ability to contract the ECM may be involved in the initiation of crimp during development. Although maintenance of the crimp in the adult is more likely due the helical arrangement of microfibrils forming kinks in the overall structure (Herchenhan, Kalson et al. 2012).

Some tendons can function not only in transferral of stress from muscle to bone but are involved in stress transferral over fibrous or bony pulleys such as the flexor tendon pulley, these tendons may be exposed to compressive loads. Different tendons are exposed to varying stresses and matrix composition is specifically tailored to stresses imposed on the tissue. For example tendons exposed to more compressive forces such as pulleys are composed of a more fibrocartilagenous substance: increased levels of aggrecan, tenascin-C and type II collagen (Perez-Castro and Vogel 1999; Martin, Mehr et al. 2003). Tendons whose properties are essential for precision tend to contain higher levels of elastic fibres, such as in the flexor tendons of the hand. Tendon composition is also variable between the regions of the tendon itself; the mid-substance contains more defined fascicles and the enthesis has an increased fibrocartilagenous phenotype (Riley 2004).

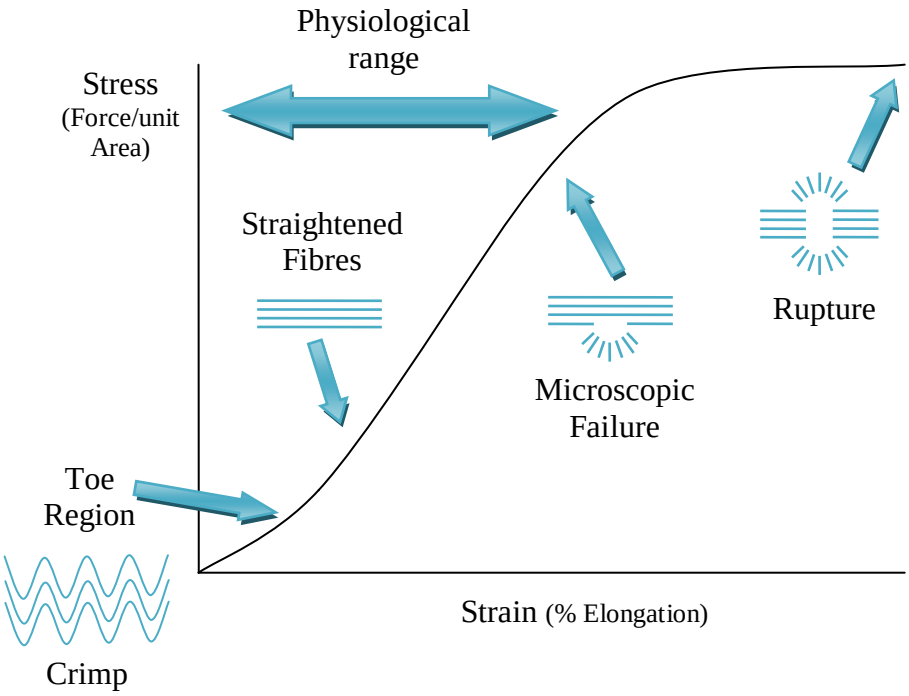

Figure 1.4. Stress strain curve. Stress, or load, is plotted against strain or percentage elongation. In the toe region fibres are exposed to very low levels of stress (force per unit area), here fibres are crimped. With increasing load, fibres straighten in a linear phase. High physiological levels of stress cause microscopic fracture and extreme levels of stress cause fibres to rupture.

1.5. TENDINOPATHY

Tendon disorders can be categorized as either acute or chronic. Acute forms of the disease involve an injury caused by either an overloading event or trauma. Subsequent to this event inflammation occurs; i.e. inflammatory cells such as mast cells, platelets and macrophages infiltrate the damaged area resulting in release of growth factors such as Transforming growth factor beta (TGFβ), connective tissue growth factor (CTGF) (Wurgler-Hauri, Dourte et al. 2007), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF). These factors attract fibroblasts which proliferate and synthesize new matrix. This new matrix is less organized and further maturation and reorganization is required to regain the strength of the tendon over an extended period (Riley 2004).

Chronic tendon disorders are thought to arise from prolonged exposure to moderate levels of strain and are often termed 'overuse' pathologies. These include a complex range of diseases whose terminology can be confusing. Chronic disease generally involves pain and insidious degeneration, onset of which cannot be associated with one traumatic event. Tendinitis and tendinosis are terms used to describe inflammatory involvement and degenerative overuse pathologies respectively (Khan, Cook et al. 1999; Khan, Cook et al. 2000). It is difficult to differentiate between the tendinosis and tendinitis in the absence of biopsy, consequently the term tendinopathy is generally used as it does not imply knowledge of the inflammatory state (Riley 2004). Chronic tendinopathies are thought to be preceded by changes in cellular expression and of the ECM which weaken the tendon (Riley 2005); however these changes are not fully understood.

Many studies examine characteristics of acute, chronic, painful and ruptured tendinopathy compared to normal tendon tissue. Differential phenotypes have been observed in each of the tendinopathies studied. Tendinopathies can show features of apparent degeneration and changes in fibroblastic activity, tenocyte apoptosis (Lian, Scott et al. 2007), GAG accumulation (Kannus and Jozsa 1991; Chard, Cawston et al. 1994; Corps, Robinson et al. 2006), calcification (Kannus and Jozsa 1991; Chard, Cawston et al. 1994; Riley, Harrall et al. 1994), lipid accumulation (Kannus and Jozsa 1991; Riley 2005), mucoid degeneration (Kannus and Jozsa 1991), hypoxia (Kannus and Jozsa 1991), increased water content (de Mos, van El et al. 2007), increased extracellular matrix turnover and a change in ECM

composition (Riley 2005). In the next section I will focus on gene regulation in tendinopathy.

1.5.1. Collagen Expression with Tendinopathy

The reduction in overall collagen content, decrease in pentosidine cross-links and the percentage increase in type III collagen in tendinopathy, suggests that protease degradation of collagen and matrix turnover is occurring (Riley, Harrall et al. 1994; Riley, Curry et al. 2002; Corps, Robinson et al. 2004; de Mos, van El et al. 2007). Other non-type I collagens that are also regulated in equine acute tendinopathies include types II, IV, X, XI (Clegg, Strassburg et al. 2007). Ireland et al reported that the increase in type III collagen was predominant in Achilles tendinopathy. The resulting higher proportion of non-type I collagen could explain the reduction in tendon strength, evident where ruptures occur (Ireland, Harrall et al. 2001). Riley *et al*, observed that type III collagen was also increased in some non-diseased samples, which may be a result of repeated microinjury. They proposed that increased collagen III incorporation may indicate an attempted healing response and may predispose tendinopathy (Riley, Harrall et al. 1994).

1.5.2. GAG and Tendinopathy

Other changes in ECM composition include increases in Glycosaminoglycans (GAG) such as hyaluronan, chondroitin sulphate and dermatan sulphate, which are seen in supraspinatus and Achilles tendinopathy (Riley, Harrall et al. 1994; Astrom and Rausing 1995). However, analysis of the Achilles ruptured tendon has shown a reduction in GAG content (Riley, Curry et al. 2002). This indicates that painful and ruptured tendon diseases are distinct in terms of GAG expression.

1.5.3. Proteoglycan and Glycoprotein regulation with Tendinopathy

Proteoglycan and glycoprotein mRNA expression is also modified with tendon pathology. Both painful and ruptured Achilles tendon mRNA analysis showed decreased expression of the proteoglycan versican (Corps, Robinson et al. 2004; Karousou, Ronga et al. 2008), another study has shown an increase in expression of versican in Achilles tendinopathy, however the study was limited to only four patients (Ireland, Harrall et al. 2001). In painful and ruptured tendon increased aggrecan and biglycan mRNA was observed (Ireland, Harrall et al. 2001; Corps, Robinson et al. 2006; Clegg, Strassburg et al. 2007; Corps, Robinson et al. 2012). In chronic tendinosis or ruptured tendons a decrease in decorin mRNA was observed (Alfredson, Lorentzon et al. 2003; Corps, Robinson et al. 2006),

however, an increase was reported in ruptured tendon (Karousou, Ronga et al. 2008). Other glycoproteins such as fibronectin, and fibrillin were reported to increase in ruptured and tendinopathic tendon (Ireland, Harrall et al. 2001; Karousou, Ronga et al. 2008; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012; Legerlotz, Jones et al. 2012). A decrease in COMP was observed in a range of tendons affected by tendinopathy (Jelinsky, Rodeo et al. 2011). Therefore substantial regulation of proteoglycans and glycoproteins is occurring in tendinopathic tendon.

Tenascin C mRNA was increased in both tendinopathic and ruptured (humans and equine) tendon (Ireland, Harrall et al. 2001; Taylor, Vaughan-Thomas et al. 2009; Jelinsky, Rodeo et al. 2011). Tenascin is reported to increase with compressive loads, therefore the increase in Tenascin C in tendinopathy may be associated with increases in compressive force (Riley, Harrall et al. 1996; Martin, Mehr et al. 2003). However, this does not necessarily mean that the tendon is responding to compressive loading. In human degenerate tendon a 300kd large isoform of tenascin C protein was increased, whereas a smaller isoform (200kD) is produced only in normal tendon (Riley, Harrall et al. 1996). This 300kD isoform may be involved in the increase of cell proliferation, cell rounding and expression of a more fibrocartilagenous phenotype (Riley, Harrall et al. 1996). In addition evidence of Tenascin C fragmentation also suggests that metalloproteinase activity is increased in degenerate tendinopathy (Riley, Harrall et al. 1996).

Many of these pathological changes are representative of a more cartilaginous phenotype. These changes include the increase in aggrecan, tenascin-C and type II collagen (Perez-Castro and Vogel 1999; Martin, Mehr et al. 2003). Clegg et al reported a more fibrocartilagenous phenotype in acute but not chronic equine superficial digital flexor tendon (SDFT) tissue (Clegg, Strassburg et al. 2007). In compressive regions of horse SDFT there was a decrease in fibrocartilagenous markers in acute tendon whereas in chronic samples the levels were similar to normal tendon. This could be due to changes in the loading pattern following injury, changing the compressive force and reducing the required stimuli to maintain fibrocartilagenous phenotype in areas normally exposed to increased compressive forces (Clegg, Strassburg et al. 2007).

1.5.4. Inflammatory cytokine regulation with Tendinopathy

Stimulation of inflammatory cytokines have also been reported in response to tendon pathology, these include IL6, IGF and TGFβ. IL6 was increased and IL6R was decreased

in both painful and ruptured tendon (6.3 and 180 fold respectively in IL6) (Jelinsky, Rodeo et al. 2011; Legerlotz, Jones et al. 2012), however there was no change in either of these genes in painful posterior tibialis tendinopathy (Legerlotz, Jones et al. 2012). The stimulation of IL6, would suggest that IL6 signalling is stimulated with tendinopathy. However due to the simultaneous decrease in IL6R, IL6 signalling may remain unchanged. In the ligament, IGF mRNA was increased in response to rupture in a rabbit model (Sciore, Boykiw et al. 1998). TGFβ protein is also increased in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002). COX2 was increased in both painful and ruptured Achilles tendon (2 and 11 fold respectively), however there was no change in painful posterior tibialis tendinopathy (Legerlotz, Jones et al. 2012). This does suggest that inflammatory pathways may be involved in tendon pathology, although the level of stimulation may be variable depending on the region of tendon pathology. There appeared to be less evidence of the stimulation of inflammatory mediators in response to posterior tibialis tendinopathy (in terms of IL6, COX2 and IL6R). And although in most respects the pathologies are very similar, this indicates that those affecting the posterior tibialis are distinct from those affecting other tendons such as the Achilles in terms of inflammatory cytokines.

1.5.5. Tendon 'marker' regulation with Tendinopathy

Although tendon does not have definitive phenotypic markers, some genes that are more commonly expressed in tendon can be used as a guide. A murine model of patella tendon injury measured an increase in scleraxis after 4-8 weeks, which returned to normal after 12 weeks (Scott, Sampaio et al. 2010). However, an equine study of acute or chronic tendon; showed that Scleraxis was not differentially regulated in either disease (Taylor, Vaughan-Thomas et al. 2009). This difference in scleraxis expression may be due to a temporal change, differential expression according to the pathology or species of origin. Immunohistochemistry study of patellar tendinopathy showed the increased expression of SOX9 protein and cells displayed a more rounded phenotype. This suggests that there is a divergence to a cartilaginous phenotype in some tendinopathic tissues (Rui, Lui et al. 2011).

1.5.6. Age and tendinopathy

Advanced Glycation End product (AGE) cross links accumulate with age, they cause reduced elasticity (Sell and Monnier 1989) and ECM turnover (Laurent 1987) by forming cross links between collagen fibre. Other age related changes include an overall modification of GAG and proteoglycan levels (Riley, Harrall et al. 1994), reduced cell

number, cellular elongation and reduced cell contacts (Ippolito, Natali et al. 1980). It has been reported that overall collagen content is not significantly modified with age although there is an overall increase in ECM density (Riley, Harrall et al. 1994). Increased type III collagen, increased collagen hydroxylysylpyridinoline cross links, lower GAG, smaller diameter collagen fibres and lower cellularity were also seen in equine SDFT aging tendon, these characteristics seen in aging horses have been suggested to be the early stages of tendon degeneration (Birch, Bailey et al. 1999). Age related changes have some similarities with the structural changes seen in tendinopathy. Therefore the age of tendon may contribute to the development of tendinopathy (Riley, Harrall et al. 1994).

Relatively little is known about the cause of this group of disorders, although the degradation of the ECM plays an important role. This may be a result of altered mechanical stress and the differential response in acute and ruptured tendon (Corps, Robinson et al. 2006).

1.6. EXTRACELLULAR MATRIX DEGRADATION IN TENDINOPATHY

A large array of proteases are expressed in prokaryotic and eukaryotic cells, these are divided into several families; including Aspartic, Cysteine, Glutamic, Metallo, Asparagine, Serine and Threonine proteases (Rawlings, Barrett et al. 2012). For example, Cathepsin proteases are a clan of proteases that form part of the Cysteine family, the majority of Cathepsins are lysosomal and are effectiveat a lower pH (Nomura and Katunuma 2005). Serine proteases are so called due to their nucleophilic Ser amino acid residue at the active site. Serine proteases are secreted as inactive zymogens and include Plasmin and Trypsin (Hedstrom 2002). Plasmin is the active form of the zymogen plasminogen, Plasmin is present in the blood and can degrade fibrin clots (Rawlings, Barrett et al. 2012). Trypsin is the active form of the zymogen trypsinogen, Trypsin hydrolyses proteins, a process known as trypsinisation (Rawlings, Barrett et al. 2012).

Metalloproteinases (Woessner 1991; Nagase and Woessner 1999; Ireland, Harrall et al. 2001), are proteases with a primary function of controlling the homeostasis of the ECM. They can break down components of the ECM so that it can be rebuilt according to current strain environment. Expression analysis has shown that a number of these molecules are regulated in tendinopathies (Fu, Chan et al. 2002; Alfredson, Lorentzon et al. 2003; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006; Clegg, Strassburg et al. 2007). Metalloproteinases are subdivided into a number of protein families; Matrix metalloproteinase's (MMPs), a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin repeats (ADAMTS). MMPs, ADAMs and ADAMTSs are important in the process of extracellular matrix degradation. Generally MMP's mediate the collagen degradation, ADAMs mediate the shedding of extracellular portions of transmembrane proteins and ADAMTSs degrade proteoglycans. Tissue inhibitors of Metalloproteinases (TIMPs) inhibit the metalloproteinase activity, therefore inhibiting the degradation of the ECM. A balance of metalloproteinase activity and matrix deposition is essential in the regulation of ECM homeostasis. Metalloproteinase expression is modulated by a wide range of cytokines, growth factors and the cellular interaction via cell surface molecules (ICAM, VLA and integrins) and mechanical strain (Nagase and Woessner 1999).

1.6.1. Matrix Metalloproteinases (MMP)

MMPs mediate the extracellular degradation of the ECM and they are active at neutral pH. They have a catalytic domain known as the metzincin motif with a zinc ion binding domain and require calcium for activity (Nagase and Woessner 1999; Bode 2003). In humans there are 23 MMPs, one system of categorisation divides them into the subgroups known as the stromelysins, gelatinases, collagenases, membrane type MMPs, matrilysins and elastin degrading proteases (See Table 1.3), classified according to their activity and structure. Collagenases, MMP1, 8 and 13 are involved in the degradation of fibrillar collagens. Gelatinases include MMP2 and 9 which degrade gelatin and non-fibrillar collagens. Stromelysins degrade type III collagen and proteoglycans (Riley 2005). They share a pro-, signal- and catalytic domains and are secreted predominantly as zymogens which are largely protease enzyme activated by the disruption of the 'cysteine switch' (a cysteine residue which ligates the zinc region in order to maintain inactivity) through cleavage of the pro-domain (Woessner 1991; Borkakoti 1998; Nagase and Woessner 1999). There are however exceptions to the rule; MMP23, 11 and all membrane bound MMPs are activated intracellularly (Nagase and Woessner 1999). Some MMPs also share a hemopexin-like domain located at the C-terminal, this is thought to be involved in the specificity; The hemopexin domain targets the enzyme to collagen, resulting in its cleavage (Nagase and Woessner 1999). Changes in MMP expression seen in tendon pathology can be associated with increased turnover and deterioration of ECM or matrix repair and synthesis (Riley, Curry et al. 2002).

Family/type	MMP	Descriptive name(s)	Known Substrates
Collagenases	**MMP-1**	Interstitial collagenase 1	Fibrillar collagens type I, II, III, VII and X; gelatins; aggrecan; link protein; entactin; tenacin; perlecan; a2-M; a1-PI; a1-antichymotrypsin; IGFBP-2, 3, 5; proIL-1b; CTGF
	MMP-8	Neutrophil collagenase, collagenase 2	collagens types I, II, III; gelatins; aggrecan; link protein; a1-PI
	MMP-13	Collagenase 3	Fibrillar collagens I, II, III, IV, IX, X and XIV; aggrecan; Fn; tenascin; osteonectin; Ln; Perlecan; CTGF; ProTGF-b; MCP-3; a1-antichymotrypsin
Gelatinases	**MMP-2**	Gelatinase A, 72 kDa gelatinase, type IV collagenase	Gelatins; Nonfibrillar collagens IV, V, VII, X and XI; Ln; Fn; elastin; aggrecan; link protein; ProTGF-b; FGF receptor I; MCP-3; IGFBP-5;proIL-1b; galectin-3; plasminogen
	MMP-9	Gelatinase B, 92 kDa gelatinase, type V collagenase	Gelatins; Non-fibrillar collagens IV and V; type III collagen; aggrecan; elastin; entactin; link protein, vitronectin; N-telopeptide of collagen I; ProTGF-b; IL-2 receptor a; Kit-L; IGFBP-3; proIL-1b; ICAM-1; a1-PI; galectin-3; plasminogen
Stromelysins	**MMP-3**	Stromelysin 1, transin	Proteoglycans; fibronectin; laminin; pro-IL-1; nonfibrillar collagens IV, IX and X; type III collagen; Aggrecan; decorin; tenascin; link protein; perlecan; IGFBP-3; proIL-1b; HB-EGF; CTGF; E-cadherin; a1-antichymotrypsin; a1-PI; a2-M; plasminogen; uPA; proMMP-1, 7, 8, 9, 13
	MMP-10	Stromelysin 2, transin-2	Aggrecan; Fn; Ln; collagens III, IV and V; link protein; ProMMP1, 8, 10
Matrilysins	**MMP-7**	Matrilysin, PUMP-1	Proteoglycans; link protein; fibronectin; Aggrecan; gelatins; Ln; elastin; entactin; collagen IV; tenascin; decorin; link protein; Proa-defensin; Fas-L; b4 integrin; E-cadherin; proTNFa; CTGF; HB-EGF; RANKL; IGFBP-3; plasminogen
	MMP-26	Matrilysin 2, endometase	Laminin; nonfibrillar collagens; gelatine; fibrin; entactin; collagen IV; Fn; fibrinogen; vitronectin; ProMMP-9; a1-PI
Elastase	**MMP-12**	Macrophage elastase, metalloelastase	Elastin; nonfibrillar collagens; aggrecan; Fn; collagen IV; osteonectin; Ln; nidogen; Plasminogen; apolipoprotein(a)
Transmembrane	**MMP-14**	MT1-MMP	Pro-MMP-2, (MMP-13), gelatin, tenascin, fibronectin, vitronectin, aggrecan; Collagens I, II and III; Ln; fibrin; Ln-5; ProMMP-2; proMMP-13; CD44; MCP-3; tissue transglutaminase
	MMP-15	MT2-MMP	Fn; tenascin; nidogen; aggrecan; perlecan; Ln; ProMMP-2; tissue transglutaminase
	MMP-16	MT3-MMP	Collagen III; Fn; gelatin; ProMMP-2; tissue transglutaminase
	MMP-24	MT5-MMP	PG; ProMMP-2
GPI anchored	**MMP-17**	MT4-MMP	Pro-MMP-2, Gelatin; fibrinogen
	MMP-25	MT6-MMP	Gelatin; collagen IV; fibrin; Fn; Ln; ProMMP-2
Miscellaneous	**MMP-11**	Stromelysin-3	α1-Proteinase inhibitor; Fn; Ln; aggrecan; gelatins; a1-PI; a2-M; IGFBP-1
	MMP-19	RASI-1	Gelatin; aggrecan; COMP; fibrin; tenascin; Collagen IV; Fn; aggrecan; Ln; nidogen; IGFBP-3
	MMP-20	Enamelysin	Amelogenin; aggrecan; COMP; gelatin
	MMP-21	xMMP	Not known
	MMP-23A	CA-MMP	Gelatin
	MMP-23B		Not known
	MMP-27	MMP-22, cMMP	Gelatin; casein
	MMP-28	Epilysin	Casein

Abbreviations: a2-M, a2-macroglobulin; a1-PI, a1-proteinase inhibitor; CA, cysteine array; cMMP: chicken MMP; COMP, cartilage oligomeric matrix protein; CTGF, connective tissue growth factor; Fas-L, Fas ligand; FGF, fibroblast growth factor; Fn, fibronectin; GPI, glycosylphosphatidylinositol; HB-EGF, heparin-binding epidermal growth factor like growth factor; IGFBP-1, insulin-like growth factor binding protein 1; ICAM-1, inter-cellular adhesion molecule 1; Kit-L, kit ligand; Ln, laminin; MBP, myelin basic protein; MCP-3, monocyte chemotactic protein-3; MMP, matrix metalloproteinase; MT, membrane-type; PG, proteoglycan; pro-IL-1, pro-interleukin 1; Pro, proteinase type; proTNF-a, pro tumor necrosis factor-a; proTGF-b, pro transforming growth factor b; ProMMP, latent MMP; PUMP, putative metalloproteinase; RANKL, receptor activator for nuclear factor k B ligand; RASI-1, rheumatoid arthritis synovium inflamed 1; TNF, tumour necrosis factor; uPA, urokinase plasminogen activator; xMMP, Xenopus MMP.

Table 1.3. Matrix Metalloproteinase family of degradative enzymes.

Adapted from Riley, 2005; Parks and Mecham, 1998; Shiomi, Lemaitre et al., 2010.

The mechanism of matrix breakdown that MMPs facilitate is fundamental in normal cell function. They are important in development, tissue desorption and remodelling, cell proliferation, migration, chemotaxis and apoptosis (Nagase and Woessner 1999). Dysfunction of these proteins can be involved in disorders such as cancer, rheumatoid arthritis, and osteoarthritis (Dudhia, Scott et al. 2007). For this reason broad spectrum inhibitors of MMPs have been used in the treatment of diseases such as cancer; MMP broad spectrum inhibitors (doxycycline and ilomostat [GM6001]) reduce pericellular matrix damage and MMP13 expression in models of tendinopathy (Arnoczky, Lavagnino et al. 2007). However these inhibitors have proved relatively ineffective and often induce side effects that resemble characteristics of tendinopathies (Drummond, Beckett et al. 1999; Tierney, Griffin et al. 1999). Although Marimistat was shown to have some anticancer effects it also induced tendinitis (musculoskeletal pain and inflammation). (Drummond, Beckett et al. 1999). This implicates MMPs as important regulators of the tendon ECM, and some activity is required to maintain a healthy tendon (Drummond, Beckett et al. 1999). More specific MMP inhibitors have shown to be ineffective in the treatment of cancer but do not induce tendinitis, this indicates that specific inhibition of MMP targets may have its place in the treatment of cancer and tendinopathy, however, more detailed study is required (Drummond, Beckett et al. 1999).

MMP expression is modified in tendon from pathological tendon compared to healthy controls. **MMP1** was increased in chronic tendinopathy and ruptured tendon compared to normal controls in a range of tendon locations (Fu, Chan et al. 2002; Riley, Curry et al. 2002; Jones, Corps et al. 2006). Other studies of tendinopathic and ruptured tendon yielded very low detection of MMP1 and/or no differential regulation of MMP1 in pathological tendon (Ireland, Harrall et al. 2001; Jelinsky, Rodeo et al. 2011). Clegg et al studied tendon injury in a horse model and found that MMP1 was up-regulated in acute tendinopathy, but were no different from the control samples in the chronic tendon (Clegg, Strassburg et al. 2007). Therefore, it is possible that the induction of MMP1 seen in acute disease is a response to matrix damage or inflammation. An increase in active MMP1 would result in the degradation of fibrillar collagens, including collagen type I (Parks and Mecham 1998; Shiomi, Lemaitre et al. 2010). As collagen type I is the major constituent of the tendon ECM (Rees, Flannery et al. 2000) this could potentially cause decreased strength and overall stability of the tissue.

Although some studies have reported no significant changes in **MMP2** with degenerate pathology (Jones, Corps et al. 2006), others have shown an induction in MMP2 mRNA and protein in tendinopathic or ruptured Achilles, posterior tibialis and supraspinatus tendon (Ireland, Harrall et al. 2001; Alfredson, Lorentzon et al. 2003; Karousou, Ronga et al. 2008; Pasternak, Schepull et al. 2008; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012). However, in tendinopathic and ruptured tendon MMP2 expression was decreased at the mRNA and protein level in supraspinatus and Achilles tendons respectively (Riley, Curry et al. 2002; Lo, Marchuk et al. 2004). Therefore levels of MMP2 expression are variable in tendinopathic and ruptured pathologies and each variety of tendon disease does not show consistent outcomes in terms of MMP2 expression.

MMP3 regulation in tendinopathy is more consistent. Studies of human Achilles and supraspinatus have recorded a significant down-regulation in mRNA, protein and activity levels of MMP3 in tendinopathic or ruptured tendon compared to normal controls (Ireland, Harrall et al. 2001; Riley, Curry et al. 2002; Alfredson, Lorentzon et al. 2003; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006). In a equine model (SPFT) MMP3 mRNA was also down-regulated (50 fold) in acute and chronic tendinopathy (Clegg, Strassburg et al. 2007). The substrate range of MMP3 includes proteoglycans (Parks and Mecham 1998; Shiomi, Lemaitre et al. 2010), consequently the observed increase of proteoglycan in pathology might therefore be explained by the decrease of MMP3. And although MMP1 and MMP3 show similarities in sequence and promoter regions, they can be regulated through separate transcription factors, which could explain their differential regulation in tendon pathologies (Buttice, Duterque-Coquillaud et al. 1996; Ireland, Harrall et al. 2001).

MMP13 mRNA up-regulation has been reported in ruptured rotator cuff tendon (Lo, Marchuk et al. 2004) and dysfunctional posterior tibialis tendon (Corps, Robinson et al. 2012), and also in an equine model of SPFT tendon, in acute and chronic forms of the disease (Clegg, Strassburg et al. 2007). However, Jones et al reported no change in MMP13 mRNA in painful or ruptured Achilles pathology (Jones, Corps et al. 2006). In addition MMP13 mRNA was undetectable in tendinopathic tissue from a mixture of tendon types (Jelinsky, Rodeo et al. 2011). This differential regulation of MMP13 with pathology may be related to temporal changes in metalloproteinase expression during the course of the disease.

Other MMPs have also been observed to be differentially regulated in tendinopathies, including MMP9, which is up-regulated at the mRNA level in ruptured Achilles tendon (Jones, Corps et al. 2006; Karousou, Ronga et al. 2008). In another Achilles tendon study, protein levels of MMP9 were increased, however, this was not reflected at the mRNA level, which may be a result of production of MMP9 occurring elsewhere (Karousou, Ronga et al. 2008). MMP19 and MMP25 were increased (mRNA) in ruptured Achilles tendon in comparison to normal controls (Jones, Corps et al. 2006; Pasternak, Schepull et al. 2008). There is a discrepancy in MMP7 regulation as mRNA was decreased in one study (Jones, Corps et al. 2006) and plasma levels increased in another (Pasternak, Schepull et al. 2008) in ruptured Achilles. This may be due to the release of MMP7 into the blood stream. In painful Achilles tendon lower expression of MMP10 and increased MMP23 was detected in comparison to normal tendon at the RNA level (Jones, Corps et al. 2006). Therefore multiple MMPs are regulated with ruptured and tendinopathic tendon. This disruption in MMP regulation may explain the decreased functionality of the tendon in these pathologies.

1.6.2. Tissue Inhibitors of Metalloproteinases

In vivo, the main regulatory proteins of the metalloproteinases are the TIMPs, of which there are four known examples (TIMP1, TIMP2, TIMP3 and TIMP4). In order to cause MMP inhibition, TIMPs bind to the MMP catalytic domain , TIMP occupation of the MMP active site results in the inhibition of substrate cleavage (Nagase and Woessner 1999). TIMPs are also involved in a number of important regulatory mechanisms including the inhibition of cellular invasion and growth (Nagase and Woessner 1999). TIMPs inhibit all MMPs (Visse and Nagase 2003), however TIMP1 is unable to inhibit the activity of MMP14 (also known as MT1-MMP) (Will, Atkinson et al. 1996). TIMP3 also inhibits ADAM and ADAMTS enzymes, such as ADAM12 (Loechel, Fox et al. 2000), and the aggrecanases, ADAMTS4 and ADAMTS5 (Kashiwagi, Tortorella et al. 2001).

Changes in the expression profile of TIMPs are evident in a number of tendon studies. TIMP1 has been found to be up-regulated both at the message (Jones, Corps et al. 2006) and protein level (Karousou, Ronga et al. 2008) in ruptured Achilles tendon, but not in acute or chronic equine tendon (Clegg, Strassburg et al. 2007). Decreased levels of TIMP2 were seen in ruptured Achilles (Jones, Corps et al. 2006) and rotator cuff tendon (Lo, Marchuk et al. 2004). However other studies have also shown TIMP2 is increased in ruptured Achilles tendon (Karousou, Ronga et al. 2008; Pasternak, Schepull et al. 2008)

and equine data (SPFT) indicated that TIMP2 was unchanged in tendinopathy (Clegg, Strassburg et al. 2007). Therefore TIMP2 regulation appears to be much more variable, studies of the same tendon pathology (i.e. ruptured Achilles) show variable results. TIMP3 and TIMP4 regulation in tendon disorders is less variable as evidence suggests that there is a decrease in the mRNA expression levels in ruptured (Lo, Marchuk et al. 2004; Jones, Corps et al. 2006), painful (Ireland, Harrall et al. 2001; Jones, Corps et al. 2006) and acute tendinopathies of the Achilles and rotator cuff (Clegg, Strassburg et al. 2007). This decrease in TIMP3 may result in increased aggrecanase activity, as TIMP3 is involved in the inhibition of ADAMTS4 and ADAMTS5 (Kashiwagi, Tortorella et al. 2001).

1.6.3. Disintegrin and Metalloproteinase domain (ADAM)

ADAMs include both transmembrane and secreted proteins involved in a number of important cellular processes including cleavage of the ectodomain of many receptors, signalling molecules, growth factors and cytokines (Edwards, Handsley et al. 2008). They are thought to be involved in differential protein folding and interaction as well as cleavage. ADAMs are made up of metalloproteinase, disintegrin and cysteine rich domains and EGF-like repeats, of which the cysteine rich domain is thought to be involved in the specific ADAM function. They are activated intracellularly by pro-protein convertases via a cysteine switch mechanism similar to the MMPs (Edwards, Handsley et al. 2008). There are 25 known ADAMs which include four pseudogenes, not all of which are proteolytically active (Table 1.4) (Shiomi, Lemaitre et al. 2010). ADAM12 is a catalytically active metalloproteinase involved in myogenesis and is expressed as two splice variants; a short form (ADAM12S) and a long, membrane bound form (ADAM12L) (Gilpin, Loechel et al. 1998; Loechel, Gilpin et al. 1998). Among other functions, ADAM12 has been shown to facilitate TGFβ signalling without the involvement of protease activity and is also the regulation of focal adhesion complexes through the interaction with integrin β3 (Kveiborg, Albrechtsen et al. 2008). ADAM12 is up-regulated in both ruptured and painful Achilles (Jones, Corps et al. 2006). Therefore the increase of ADAM12 may result in the dysregulation of TGFβ signalling or cell communication. ADAM8 is a sheddase, it is known to cleave L-selectin and may be involved in neutrophil function in active sites of inflammation (Gomez-Gaviro, Dominguez-Luis et al. 2007). ADAM8 was also increased in ruptured Achilles (Jones, Corps et al. 2006). However the role of ADAM8 in tendinopathy remains to be determined. No other studies of pathology have studied the regulation of ADAMs.

ADAM	Other names	Proteinase/non-proteinase? Splice varient?	Potential function	Examples of substrates	Integrin binding
ADAM1	PH-30α, Fertilin-α	Pseudogene	ND	Unknown	ND
ADAM2	PH-30β, Fertilin-β	NP	ND	Sperm/egg binding/fusion	α4β1, α6β1, α9β1
ADAM3	Cyritestin, tMDC, CYRN	Pseudogene	ND	Unknown	ND
ADAM5	Tmdc II	Pseudogene	ND	Unknown	ND
ADAM6	tMDC IV	Pseudogene	ND	Unknown	ND
ADAM7	EAP I, GP-83	NP	ND	Unknown	α4β1, α4β7, α9β1
ADAM8	MS2 (CD156)	P	Sheddase, neutrophil infiltration	CD23, proTNFa, RANKL	ND
ADAM9	MDC9, MCMP, Meltrin-g	P, Secreted form	Sheddase, cell migration	ProHB-EGF, TNF-p75 receptor, APP, fibronectin, gelatin,	α2β1, α6β1,α6β4, α9β, αVβ5
ADAM10	MDAM, Kuzbanian	P	Sheddase, development, angiogenesis	ProTNF-a, collagen IV, gelatin Myelin basic protein, Delta, APP, L1, CD44, proHB-EGF, Notch, Delta-like 1, Jagged, N-cadherin, E-cadherin, VE-cadherin, Ephrin A2, Ephrin A5 Fas-L, IL6R	ND
ADAM11	MDC	NP, Secreted form	Tumor suppressor gene?	Unknown	ND
ADAM12	Meltrin-a, MCMP, MLTN, MLTNA	P, Secreted form Sheddase, myogenesis,	adipogenesis	ProHB-EGF, IGFBP-3 and 5, pro-epiregulin collagen IV, gelatin, fibronectin	α4β1, α7β1, α9β1
ADAM15	Metargidin, MDC15, AD56, CR II-7	P, Cytoplasmic form	Arteriosclerosis, angiogenesis	Collagen IV, gelatin aVb3, a4b1,	α5β1, α9β1
ADAM17	TACE, Csvp	P	Sheddase, heart development	ProTNF-a, proTGF-a, TNF-p75 receptor, ErbB4, TRANCE, proHB-EGF, proamphiregulin, proepiregulin, APP, IL6R, CD44, L-selectin	α5β1
ADAM18	ADAM27, tMDC III	NP	ND	Unknown	ND
ADAM19	Meltrin-b, FKSG34	P, N-terminal	Sheddase, formation of neuron and cardiovascular organs	Proneuregulin, RANKL	α4β1, α5β1
ADAM20		P	Formation of sperm	Unknown	ND
ADAM21	ADAM31	P	ND	Unknown	ND
ADAM22	MDC2	NP, Cytoplasmic	ND	Unknown	ND
ADAM23	MDC3	NP	ND	Unknown	αVβ3
ADAM28	e-MDC II, MDC-Lm, MDC-Ls	P, Secreted form	Growth factor metabolism	Myelin basic protein, IGFBP-3, CD23	α4β1, α4β7, α9β1
ADAM29	svph 1	NP	ND	Unknown	ND
ADAM30	svph 4	P	ND	Unknown	ND
ADAM32	AJ131563	NP	ND	Unknown	ND
ADAM33	NA	P	Genetically related to bronchial asthma	APP, KL-1, insulin B chain	α4β1, α5β1, α9β1
ADAMDEC1	NA	P	ND	Unknown	ND

APP, amyloid precursor protein; Fas-L, Fas Ligand; HB-EGF, heparin-binding epidermal growth factor; IGFBP-3, insulin-like growth factor binding protein-3; IL6R, interleukin-6 receptor; KL-1, Kit-ligand-1; NA, not applicable; ND, no data; RANKL, receptor activator of nuclear facto kB ligand; TGF, transforming growth factor; TNF, tumor necrosis factor; TRANCE, TNF-related activation induced cytokine.

Table 1.4. The ADAM family of Metalloproteinases
Adapted from Shiomi, Lemaitre et al., 2010.

1.6.4. A Disintegrin and Metalloproteinase domain with Thrombospondin motifs (ADAMTS)

There are 19 ADAMTS proteases currently known (Table 1.5); they are synthesized as zymogens and activated through the removal of the pro-domain (Wang, Tortorella et al. 2004). However, ADAMTS7 and ADAMTS13 show catalytic activity when the pro-domain is still attached (Majerus, Zheng et al. 2003; Somerville, Longpre et al. 2004). ADAMTS2, ADAMTS3 and ADAMTS14 are involved in the processing of pro-collagen (Colige, Beschin et al. 1995; Colige, Li et al. 1997; Fernandes, Hirohata et al. 2001; Wang, Lee et al. 2003). ADAMTS 1, 4, 5 and 15 are reported to possess aggrecanase activity (Porter, Clark et al. 2005). ADAMTS4 and ADAMTS5 (also known as aggrecanase 1 and 2 respectively) are the most well characterised and are also involved in the degradation of versican and brevican (Porter, Clark et al. 2005). ADAMTS5 is reported to posses 1000 fold more aggrecanase activity than ADAMTS4, ADAMTS5 is therefore considered to be the major aggrecanase (Gendron, Kashiwagi et al. 2007). ADAMTS7 has no known substrate, although it has been established that it does not possess aggrecanase activity (Porter, Clark et al. 2005). ADAMTS13 is also known as von Willebrand factor cleavage protease and is least like the other ADAMTSs due to its possession of two CUB domains (Zheng, Chung et al. 2001; Porter, Clark et al. 2005). ADAMTS13 possesses an RGDS domain within the cysteine rich domain that could be involved in integrin binding (Zheng, Chung et al. 2001). ADAMTS13 has only one known substrate, which is von Willebrand factor (Porter, Clark et al. 2005).

ADAMTS5 was decreased in acute (Clegg, Strassburg et al. 2007) and painful (Jones, Corps et al. 2006) but remained unchanged in equine chronic tendinopathy (Clegg, Strassburg et al. 2007). However, an increase in ADAMTS5 is also reported in dysfunctional posterior tibialis tendon (Corps, Robinson et al. 2012). Clegg et al showed no change in ADAMTS4 in any phenotype in equine tendon pathology (Clegg, Strassburg et al. 2007), however human studies demonstrated an increase in ruptured tendon (Jones, Corps et al. 2006; Corps, Jones et al. 2008; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012). In addition, Jelinsky et al reported no significant change in ADAMTS genes in chronic tendinopathy, however this study groups together many different pathological

phenotypes and may not be a good representation of any of these (Jelinsky, Rodeo et al. 2011). Therefore differences in the pathology and species could explain this variation in ADAMTS4 and ADAMTS5 gene expression. The increase in ADAMTS4 in ruptured tendinopathy may be associated with increased levels of proteoglycan degradation. Jones et al reported that ADAMTS2 and ADAMTS3 are increased in painful tendon (Jones, Corps et al. 2006); as ADAMTS2 and ADAMTS3 are involved in pro-collagen processing, pro-collagen processing may be increased in painful tendon (Colige, Beschin et al. 1995; Colige, Li et al. 1997; Fernandes, Hirohata et al. 2001; Wang, Lee et al. 2003). ADAMTS7 and ADAMTS13 were decreased in ruptured tendon (Jones, Corps et al. 2006); however how the regulation of these genes is related to the tendon pathology remains unknown due to limited substrate knowledge.

From these data we can see that a number of metalloproteinases are regulated in tendon pathology, however, there is a distinct profile for each classification of tendinopathy such as painful and ruptured tendons and also the specific tendon type (Jones, Corps et al. 2006). A potential mechanism of tendon rupture may be the dysregulation of proteases, destabilizing the ECM, predisposing the tendon to rupture (Kannus and Jozsa 1991; Riley, Curry et al. 2002; Jones, Corps et al. 2006; Pasternak, Schepull et al. 2008). Further understanding of how these metalloproteinases and TIMPs are regulated in these pathologies may lead to successful treatment of these disorders.

Protease name	Alternative name (s)	Known substrates
ADAMTS-1	METH-1; aggrecanase-3; C3-C5; KIAA1346	Aggrecan; versican V1 (binds heparin)
ADAMTS-2	pro-collagen I N-proteinase; hPCPNI ; PCINP	Procollagen I, II and III N-propeptides
ADAMTS-3	KIAA0366	Procollagen II N-propeptide
ADAMTS-4	KIAA0688; Aggrecanase-1; ADMP-1	Aggrecan; brevican; versican V1; fibromodulin; decorin;carboxymethylated transferring; fibronectin
ADAMTS-5	aggrecanase-2; ADAMTS11; Aggrecanase-2, ADMP-2	Aggrecan; versican; brevican
ADAMTS-6		Unknown
ADAMTS-7		Cartilage Oligomeric Matrix Protein (COMP)
ADAMTS-8	METH-2	Aggrecan (inhibits angiogenesis)
ADAMTS-9	KIAA1312	Aggrecan; versican
ADAMTS-10		Unknown
ADAMTS-12		Unknown
ADAMTS-13	vWFCP, C9orf8	von Willebrand factor
ADAMTS-14		Procollagen I N-propeptide
ADAMTS-15		Aggrecan
ADAMTS-16		Aggrecan
ADAMTS-17	FLJ32769; LOC123272	Unknown
ADAMTS-18	ADAMTS21; HGNC:16662	Aggrecan
ADAMTS-19		Unknown
ADAMTS-20		Versican; Aggrecan

Table 1.5. ADAMTS Family of Metalloproteases
Adapted from Porter, Clark et al., 2005; Shiomi, Lemaitre et al., 2010.

1.7. MECHANICAL STRAIN

Moderate mechanical is said to be important in the functioning of normal tissue homeostasis (Ingber 1997; Arnoczky, Lavagnino et al. 2008). However, high levels of mechanical strain have been implicated as a causative factor of tendinopathies (Dudhia, Scott et al. 2007). Compared to the number of similar studies on various other tissue fibroblasts; such as cardiac (Brancaccio, Hirsch et al. 2006; Gupta and Grande-Allen 2006), endothelial (Sasamoto, Nagino et al. 2005) and bone cells (Salter, Wallace et al. 2000), relatively few studies have focused on the effects of mechanical strain upon tendon cells. In non-tendon tissues mechanical strain is reported to increase cellular proliferation (Zhang, Li et al. 2003); to modulate ECM molecules including metalloproteinases, collagen, glycoproteins, GAGs and growth factors (Gupta and Grande-Allen 2006); to accelerate the process of healing as compared to prolonged rest periods (Buckwalter 1996) and regulate MMP expression (Meng, Mavromatis et al. 1999). Tendon studies have shown that physiological levels of cyclic strain (~5%) induce anabolic regulation of collagen related molecules. As a result, strain maintains the ECM and induces modification of GAG content and cell proliferation (Screen, Shelton et al. 2005). In summary MMPs are important in ECM homeostasis, they are differentially regulated in pathological tendon (Ireland, Harrall et al. 2001; Riley, Curry et al. 2002; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006; Clegg, Strassburg et al. 2007; Karousou, Ronga et al. 2008; Pasternak, Schepull et al. 2008) and mechanical strain can modulate their expression (see below). Therefore mechanical strain regulation of MMPs may play a role in the development of tendinopathy.

Existing studies of metalloproteinase expression with mechanical load include multiple systems with different types of straining regime. Cyclic loading regimes are used in an effort to replicate physiological movement. However, static loading regimes have also been studied, which seem much less relevant to physiological movement (For full list of references see figures 1.6-1.10). There is a high level of variation in the length of straining regime used as well as the measurement of the strain itself (i.e. % strain or MPa); therefore it is difficult to compare gene expression between these studies. Some studies also measure gene expression some time after the straining has stopped (Tsuzaki, Bynum et al. 2003),

therefore consequent changes in gene expression may be more representative of unloaded tendon. The majority of studies have used tendon fascicles (Asundi and Rempel 2008), however in some cases cells are extracted from tissues and mechanically loaded upon stretching devises such as the flexcell^{TM} or the flexible silicone membrane system developed by James Wang's group (Tsuzaki, Bynum et al. 2003; Yang, Im et al. 2005). There are obvious limitations to these studies as the cells are not in 3D culture (Taylor, Vaughan-Thomas et al. 2009).In vivo studies of partial width laceration in ovine tendon may not fully represent stress deprivation and high level strain, as the laceration itself may induce changes not related to mechanical loading (Smith, Sakurai et al. 2008). Human in vivo studies of metalloproteinase regulation are limited as the measurements are taken from dialysate from around the tendon, therefore the metalloproteinase's measured here may not be produced in the tendon at all (Koskinen, Heinemeier et al. 2004). However, taken together these studies are beginning to paint a picture of how mechanical loading regulates metalloproteinase expression.

1.7.1. MMP1mRNA regulation with mechanical load

Static and cyclic strains, as well as fluid flow, have been shown to regulate MMP mRNA expression (See tables 1.6-1.11 for summary and references). The regulation of MMP1 depends on the type and duration of strain. In vitro studies of human and rabbit tendon cells (flexor digitorum profundus and Achilles respectively) in monolayer showed that a cyclic strain regime of up to 5% (at 1Hz for 2 hours and 18 hours rest or 0.33Hz for 6 hours) had little effect upon MMP1 expression (Archambault, Tsuzaki et al. 2002; Tsuzaki, Bynum et al. 2003). In addition in ex vivo fascicle studies of rabbit flexor tendon, there was also no change in MMP1 expression in response to 2-6MPa cyclic strain at 0.45Hz for 18 hours (Asundi and Rempel 2008). Increased strain levels as a result of in vivo partial width laceration in an ovine exercise model showed no change in MMP1 expression after 4 weeks (Smith, Sakurai et al. 2008). However, cells from human patellar tendon seeded into grooved silicone dishes showed an increase in MMP1 expression in response to 4 or 8% at 1Hz after 4 hours (Yang, Im et al. 2005). The aforementioned studies where MMP1 was unchanged in response to mechanical load are of lower frequency or duration, therefore the strain response may only occur once a threshold has been reached. However this level may never be reached in vivo (see table 1 for a summary of MMP1 changes with strain).

The induction of MMP1 in response to cyclic strain in the study of human cells seeded onto a silicone membrane shows a response unlike any of the rodent studies (Yang, Im et al. 2005). Despite the use of human cells in this study, it may not be physiologically relevant as tendon cells grown in monolayer behave differently to those in 3D culture (Taylor, Vaughan-Thomas et al. 2009), the grooved culture dish used in this study prevents transverse cellular interaction. Therefore the responses Yang *et al* have observed in the monolayer culture system are likely to be very different to those observed in vivo. In addition the absence of 3D culture means that cells are directly exposed to the loading regime imposed upon them; as opposed to cells in 3D culture of tendon fascicles, where strain is dissipated by the surrounding matrix. Therefore this experiment may represent the response to a higher loading regime. All other studies of MMP1 regulation with strain are of non-human derived tissue. Thus existing studies of MMP1 expression in response to load may not represent the in vivo situation. Detailed in vivo and in vitro (3D culture) study of human tenocytes in response to mechanical load is important to follow up this preliminary research.

Static loading also induces differential modulation of MMP1 compared to unstrained controls. Stimulation of ex vivo rabbit tendon fascicles with 6MPa static load induced an increase in MMP1 expression after 20 hours. However with a lower magnitude of strain (2 or 4 MPa) there was no change in MMP1 expression (Asundi and Rempel 2008). Static loading therefore appears to have a response threshold similar to cyclic loading, like cyclic regimes higher magnitudes of loading induce a catabolic effect.

During tendon loading, displacement of the ECM can induce the movement of fluid within the tissue (Archambault, Elfervig-Wall et al. 2002). Fluid flow induces an increase in MMP1 expression after 9 hours at 1dyne/cm^2 (Archambault, Elfervig-Wall et al. 2002). However, physiological levels of fluid flow have not been measured in response to mechanical load (Archambault, Elfervig-Wall et al. 2002). Therefore we cannot determine whether this level of fluid flow is physiologically relevant. Hence it is not possible to directly compare this response to the static or cyclic loading regime. Nevertheless, this level of fluid flow induces a catabolic response similar to that of 6MPa static load in a rabbit tendon study (Asundi and Rempel 2008). Therefore 1dyne/cm^2 fluid flow may develop in response to approximately 6MPa static load in vivo.

Stress deprivation induces an increase in MMP1 expression in an in vivo ovine study (Smith, Sakurai et al. 2008). Taken together these data from a range of loading regimes suggests that either the lack of or an excess of tension (above a certain threshold) induces the up-regulation of MMP1 expression. There is similarity between static and cyclic loading in terms of MMP1 regulation, although no direct comparison could be made due to differences in loading magnitude and duration. Sensitivity of cells to loading may also depend on species. As rabbit studies did show more catabolic response to load in terms of MMP1 expression (Archambault, Elfervig-Wall et al. 2002; Asundi and Rempel 2008).

Type of mechanical stimulation		Cell or tissue origin	MMP1 regulation	Reference
Cyclic strain	In vitro - cells seeded onto silicone dishes	Human patellar fibroblasts	MMP1 ↑ with 4 or 8% strain at 0.5Hz for 4 hours	(Yang, Im et al. 2005)
	In vitro – cells seeded into bioflex culture plates	Human flexor digitorum profundus	MMP unchanged with 3.5% strain at 1Hz (2 hours followed by 18 hours rest)	(Tsuzaki, Bynum et al. 2003)
		Rabbit Achilles paratenon (New Zealand Male white rabbits)	MMP1 was undetected even in control cultures (5% Elongation at 0.33Hz for 6 hours)	(Archambault, Tsuzaki et al. 2002)
	Ex vivo – flexor tendon fascicles	Rabbit flexor tendon	MMP1 unchanged with 2,4 or 6MPa at 0.45Hz for 18 hours	(Asundi and Rempel 2008)
In vivo exercise	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP1 unchanged with increased strain after 4 weeks	(Smith, Sakurai et al. 2008)
Static strain	Ex vivo – Loading of flexor tendon fascicles	Rabbit flexor tendon	MMP1 unchanged with 2 or 4 MPa MMP1 ↑ with 6MPa For 20 hours	(Asundi and Rempel 2008)
Fluid Flow	In vitro – cells seeded onto collagen bonded glass cover slips	Rabbit Achilles paratenon	MMP1 ↑ with 9 hours fluid flow at 1dyne/cm^2	(Archambault, Elfervig-Wall et al. 2002)
Stress Deprivation	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP1 ↑ with stress deprivation after 4 weeks	(Smith, Sakurai et al. 2008)

Table 1.6. MMP1 mRNA regulation with mechanical load

1.7.2. MMP2 mRNA regulation with mechanical load

A human in vivo study showed proMMP2 was decreased in Achilles peritendinous tissue dialysate after 1 day and increased after 3 days after 1 hour of intense uphill running (Koskinen, Heinemeier et al. 2004). In vivo studies of loading in rodent and ovine tissue have shown that after prolonged periods of high-level load, MMP2 mRNA is increased (Heinemeier, Olesen et al. 2007; Smith, Sakurai et al. 2008; Sun, Andarawis-Puri et al. 2010). Therefore prolonged high level loading may induce MMP2 expression; which can be associated with matrix reorganisation. Legerlotz et al showed no change in MMP2 expression after 12 weeks treadmill running inrat Achilles (Legerlotz, Schjerling et al. 2007). This difference in response could be due to strain magnitude or due to differences in species. However it is difficult to compare the level of strain that the cells undergo in different models, due to the differential makeup of the tendon. Stress deprivation of rat tendon fascicles induced an increase in MMP2 expression in an ex vivo model (Leigh, Abreu et al. 2008). This evidence indicates that high level or lack of mechanical load increase MMP2, however lower level stress or shorter strain period has no affect upon MMP2 mRNA expression. Study of different loading regimes is required to further understand MMP2 regulation in human specimens (For a summary of changes with strain see table 2).

Type of mechanical stimulation		Cell or tissue origin	MMP2 regulation	Reference
In vivo exercise	1 hour of uphill treadmill running	Human, healthy young males	proMMP2 ↓ after 1 day and ↑ after 3 days (protein)	(Koskinen, Heinemeier et al. 2004)
	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP2 mRNA ↑ with increased strain after 4 weeks	(Smith, Sakurai et al. 2008)
	In vivo - 12 weeks treadmill running	Achilles tendon from Sprague Dawley Rats	MMP2 mRNA unchanged with strain	(Legerlotz, Schjerling et al. 2007)
	In vivo - sub failure fatigue loading	Rat patellar tendon from female Sprague Dawley rats	MMP2 mRNA ↑ 1 and 7 days post 100 load cycle MMP2 mRNA ↑ 7 days post 7200 load cycle but **not** after 1 day	(Sun, Andarawis-Puri et al. 2010)
	In vivo - electrical muscle stimulation	Female Sprague Dawley Rat Achilles tendon	MMP2 mRNA ↑ with concentric and isometric exercise but not eccentric after 4 days	(Heinemeier, Olesen et al. 2007)
Stress Deprivation	Ex vivo – unloading of tendon fascicles	Rat tail tendon from Sprague Dawley Rats	MMP2 ↑ with 6 hours stress deprivation	(Leigh, Abreu et al. 2008)

Table 1.7. MMP2 mRNA and protein regulation with mechanical load

1.7.3MMP3 mRNA regulation with mechanical load

In human and rodent in vitro cell culture studies of cyclic strain, MMP3 mRNA was increased with 2-6 hours of 3.5-5% strain at 0.33-1Hz followed by 18 hours of rest, however this was not reflected at the protein level (Archambault, Tsuzaki et al. 2002; Tsuzaki, Bynum et al. 2003). This response may be a result of the 18 hour rest period rather than a response to the strain itself. Other studies that have looked at MMP3 expression directly after loading have shown a decrease in expression. For example a study of ex vivo flexor tendon of the rabbit showed MMP3 was decreased in response to 4MPa strain at 0.45Hz after 18 hours, and was unchanged with increased magnitude (Asundi and Rempel 2008). In rat tail tendon studies MMP3 mRNA was increased after 10 minutes of loading but was unchanged after 1 or 24 hours (3% loading upon a static load of 2% at 1Hz) (Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010). This differential response may be due to the underlying static load used in this study (for a summary of MMP3 changes with strain see table 3).

Fatigue loading of rat tail tendon induced an increase in MMP3 expression in response to a short loading cycle, whereas a longer strain regime had no effect upon MMP3 expression (Sun, Andarawis-Puri et al. 2010). Fluid flow at 1dyne/cm^2 also increased MMP3 mRNA expression (Archambault, Elfervig-Wall et al. 2002). This data suggests that MMP3 regulation depends of the magnitude and duration of loading. Shorter duration and higher magnitude induce a more catabolic response, whereas longer and lower levels of strain induce an anabolic response. Stress deprivation induced an increase in MMP3 mRNA after 8 hours (Leigh, Abreu et al. 2008). Therefore the lack of tension also increases MMP3 expression.

Type of mechanical stimulation		Cell or tissue origin	MMP3 regulation	Reference
Cyclic strain	In vitro – cells seeded into bioflex culture plates	Human flexor digitorum profundus	MMP3 mRNA ↑ with 2 hours 3.5% strain at 1Hz (followed by 18 hours rest)	(Tsuzaki, Bynum et al. 2003)
		Rabbit Achilles paratenon (New Zealand Male rabbits)	MMP3 mRNA ↑ with 5% Elongation at 0.33Hz for 6 hours + 18 hours rest unchanged with strain at the protein level	(Archambault, Tsuzaki et al. 2002)
	Ex vivo – Tendon fascicles	Rabbit flexor tendon	MMP3 mRNA ↓ with 4MPa strain at 0.45Hz Unchanged at 5MPa after 18 hours	(Asundi and Rempel 2008)
		Rat tail tendon from male Wister rats	MMP3 mRNA ↑ at 10 minutes of 3% cyclic strain superimposed on a 2% static load	(Maeda, Shelton et al. 2009)
			MMP3 mRNA unchanged at either 1 or 24 hours with 3% cyclic strain superimposed on a 2% static load (4 hour incubation in medium before strain protocol) at 1Hz	(Maeda, Fleischmann et al. 2010)
In vivo exercise	In vivo - sub failure fatigue loading	Rat patellar tendon from female Sprague Dawley rats	MMP3 mRNA ↑ 1 and 7 days post 100 load cycle MMP3 mRNA unchanged post 7200 load cycle	(Sun, Andarawis-Puri et al. 2010)
Fluid flow	In vitro – cells seeded onto collagen bonded glass coverslips	Rabbit Achilles paratenon	MMP3 mRNA ↑ with 9 hours fluid flow at 1dyne/cm^2	(Archambault, Elfervig-Wall et al. 2002)
Stress Deprivation	Ex vivo – unloading of tendon fascicles	Rat tail tendon from Sprague Dawley Rats	MMP3 mRNA ↑ with 8 hours stress deprivation	(Leigh, Abreu et al. 2008)

Table 1.8. MMP3 regulation with mechanical load

1.7.4. MMP9 mRNA regulation with mechanical load

Studies of in vivo exercise have measured MMP9 expression. Humans showed increased levels of proMMP9 in the tendon tissue dialysate, 1 and 3 days after 1 hour of uphill treadmill running (Koskinen, Heinemeier et al. 2004). Studies of partial width laceration and sub failure fatigue loading showed no change in MMP9 mRNA expression with up to 4 weeks of mechanical load, however stress deprivation induced an increase in MMP9 mRNA (Smith, Sakurai et al. 2008; Sun, Andarawis-Puri et al. 2010). In summary, human studies suggest that MMP9 induction may occur in response to mechanical load in the surrounding tendon area. However in ovine and rodent studies MMP9 is not induced at the mRNA level in response to load in tendon cells and lack of tension induces a catabolic effect (for a summary of MMP9 changes with strain see table 4).

Type of mechanical stimulation		Cell or tissue origin	MMP9 regulation	Reference
In vivo exercise	1 hour of uphill treadmill running	Human, healthy young males	proMMP9 ↑ after 1 day and 3 days (protein)	(Koskinen, Heinemeier et al. 2004)
	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP9 mRNA unchanged with excessive strain after 4 weeks	(Smith, Sakurai et al. 2008)
	In vivo - sub failure fatigue loading	Rat patellar tendon from female Sprague Dawley rats	MMP9 mRNA was unchanged with 100 or 7200 load cycle after 1 or 7 days	(Sun, Andarawis-Puri et al. 2010)
Stress Deprivation	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP9 mRNA ↑ with stress deprivation after 4 weeks	(Smith, Sakurai et al. 2008)

Table 1.9. MMP9 regulation with mechanical strain

1.7.5. MMP13 mRNA expression with mechanical load

In vivo and ex vivo rat tail and patellar tendon studies have shown that lower magnitudes (<6%) of cyclic strain decrease the level of MMP13 mRNA expression (Arnoczky, Lavagnino et al. 2008; Gardner, Arnoczky et al. 2008; Sun, Li et al. 2008; Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010). In studies of higher level mechanical loading such as repeated cyclic loading of rat patellar tendon until the fascicles reached 1.7% elongation (Sun, Li et al. 2008) or partial width laceration in ovine tendon (Smith, Sakurai et al. 2008), MMP13 was increased. Intermittent in vitro hydrostatic pressure as well as cyclic loading induced a magnitude dependant response. Moderate load (1.5MPa hydrostatic pressure or 0.6% elongation) reduced the level of MMP13, however high level load (7.5MPa, 1.7% elongation) increased MMP13 mRNA expression (Sun, Li et al. 2008). This indicates that the strain response of MMP13 mRNA is magnitude sensitive. Sub failure fatigue studies showed that low repetition of high level strain induced an increase in MMP13 expression, however in response to a longer loading regimen MMP13 was decreased (Sun, Andarawis-Puri et al. 2010). Therefore low level repetition induces a more catabolic response in MMP13 expression than in high level repetition studies. Sun and colleagues proposed that prolonged high level loading induced an adaptive response rather than a catabolic response (Sun, Andarawis-Puri et al. 2010). This indicates that tendon cells may be able to increase tendon strength in response to long term loading by reducing the level of ECM degradation, although confirmation that this is actually occurring at the protein level is necessary. Stress deprivation of tendon fascicles increased MMP13 expression in a number of rat tail tendon studies (Lavagnino, Arnoczky et al. 2006; Arnoczky, Lavagnino et al. 2007; Arnoczky, Lavagnino et al. 2008; Leigh, Abreu et al. 2008). In summary high level and absence of tension induces a catabolic effect whereas lower level and/or prolonged strain produces an anabolic response in terms of MMP13 expression (for summary see table 5).

Type of mechanical stimulation		Cell or tissue origin	MMP13 regulation	Reference
Cyclic strain	In vivo - loading of tendon fascicles	Rat patellar tendon from adult female Sprague Dawley rats	MMP13 mRNA, protein and activity ↓ with loading at 1Hz until 0.6% elongation, ↑with loading at 1Hz until 1.7% elongation	(Sun, Li et al. 2008)
	Ex vivo – loading of tendon fascicles	Rat tail tendon	MMP13 ↓ with strain (1-6% at 0.017Hz)	(Gardner, Arnoczky et al. 2008)
			MMP13 ↓ with 1% strain at 24 hours	(Arnoczky, Lavagnino et al. 2008)
		Rat tail tendon from male Wister rats	MMP13 mRNA ↓ at 24 hours of 3% cyclic strain super-imposed on a 2% static load	(Maeda, Shelton et al. 2009)
			MMP13 mRNA ↓ 1 and 24 hours with 3% cyclic strain superimposed on a 2% static load (4 hour incubation in mediumbefore strain protocol)	(Maeda, Fleischmann et al. 2010)
Hydrostatic pressure	In vitro – intermittent hydrostatic pressure	Clonal cells derived from rat patellar tendon	MMP13 mRNA, protein and activity ↓ with 1.5MPa at 1Hz ↑with 7.5 MPa at 1Hz	(Sun, Li et al. 2008)
In vivo exercise	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP13 mRNA ↑ with increased strain and strain deprivation after 4 weeks	(Smith, Sakurai et al. 2008)
	In vivo - sub failure fatigue loading	Rat patellar tendon from female Sprague Dawley rats	MMP13 mRNA ↑ 1 and 7 days post 100 load cycle MMP13 mRNA ↓ 7 days post 7200 load cycle	(Sun, Andarawis-Puri et al. 2010)
Stress Deprivation	Ex vivo – isolated fibrillar damage	Rat tail tendon	MMP13 mRNA and protein ↑ at sites of microdamage	(Lavagnino, Arnoczky et al. 2006)
	Ex vivo – unloading of tendon fascicles		MMP13 mRNA, protein and activity ↑ with 7 days stress deprivation	(Arnoczky, Lavagnino et al. 2007)
			MMP13 mRNA ↑ with 24 hours stress deprivation	(Arnoczky, Lavagnino et al. 2008)
			MMP13 mRNA ↑ with 8 hours stress deprivation	(Leigh, Abreu et al. 2008)
	In vivo partial width laceration	Sheep purebred merino wethers, infraspinatus tissue	MMP13 mRNA ↑ with stress deprivation after 4 weeks	(Smith, Sakurai et al. 2008)

Table 1.10. MMP13 Regulation with mechanical strain

1.7.6. Rat Collagenase

Sprague Dawley Rats are often used in the study of tendon with respect to mechanical load. However rat species do not express MMP1 and MMP13 as they are known in humans. They do express what has been termed Rat collagenase, which is most closely related in terms of sequence homology to human MMP13 (Mitchell, Magna et al. 1996). However, some research groups have published data which describes the regulation of MMP1 in Rat tendon in response to mechanical load (Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004). However this description is not a true representation as Rat collagenase is less homologous to human MMP1 than it is to human MMP13 (Mitchell, Magna et al. 1996). Therefore this section describes the regulation of rat collagenase separate from the other MMPs. In an in vivo study of rat tendon fascicles 1% strain at 1Hz decreased rat collagenase expression after 24 hours (Lavagnino, Arnoczky et al. 2003). This study also showed that not only the amplitude but the frequency of strain was also important in instigating a response in terms of rat collagenase regulation (Lavagnino, Arnoczky et al. 2003). Another study of rat tail tendon showed that rat collagenase was decreased with up to 6MPa static load after 24 hours (Arnoczky, Tian et al. 2004). Studies of stress deprivation in rat tail tendon, over a period of 24 hours they observed an increase in MMP1 mRNA expression (Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004). Therefore, it is clear that Rat collagenase is decreased in response to moderate mechanical strain and increased with stress deprivation (for a summary of rat collagenase changes see table 6).

Type of mechanical stimulation		Cell or tissue origin	Rat Collagenase regulation	Reference
Cyclic strain	Ex vivo – flexor tendon fascicles	Rat tail tendon	Rat Collagenase ↓ with 1% strain at 1 Hz for 24 hours	(Lavagnino, Arnoczky et al. 2003)
Static strain	Ex vivo – Loading of rat tail tendon	Rat tail tendon from Sprague Dawley rats	Rat Collagenase ↓ with 2-6MPa for 24 hours	(Arnoczky, Tian et al. 2004)
Stress Deprivation	Ex vivo – Rat tendon fascicles	Rat tail tendon	Rat Collagenase ↑ with 24 hours stress deprivation	(Lavagnino, Arnoczky et al. 2003)
		Rat tail tendon from Sprague Dawley Rats	Rat Collagenase ↑ with 24 hours stress deprivation	(Arnoczky, Tian et al. 2004)

Table 1.11. Rat Collagenase mRNA and protein regulation with mechanical load

1.7.7. Other MMPs regulated with mechanical load

Previous studies have observed the regulation of MMP14 mRNA in response to mechanical loading. After a short period (100 cycles) of straining, MMP14 was increased (7 days after the strain period), however, after a longer period (7200 cycles) of strain MMP14 was decreased (1 day after the strain period) (Sun, Andarawis-Puri et al. 2010). In an ovine study involving partial width laceration of the infraspinatus tendon, MMP14 was increased in stress deprived areas but unchanged in areas of increased loading (Smith, Sakurai et al. 2008). Therefore MMP14 mRNA is sensitive to mechanical load and duration determines the direction of regulation. Maeda *et al*, also looked at MMP10, MMP12, MMP16 and MMP23 using microarray, however they showed no change in any of these genes in response to 3% cyclic strain superimposed on a static strain of 2% (Maeda, Fleischmann et al. 2010). Therefore not all of the metalloproteinase genes have been measured in response to mechanical loading and protein quantification is limited. A full investigation of the regulation of metalloproteinase genes is required to further understand how matrix turnover is regulated in response to mechanical load.

1.7.8. ADAMTS and TIMP regulation with mechanical load

Mechanical stimulation in rat tendon induced an increase in mRNA and protein expression of TIMPs 1 and 2 (Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007). The increase of TIMP expression is suggestive of a more anabolic phenotype, however functions of TIMP2 include the activation of proMMP2 (Wang, Juttermann et al. 2000). Therefore the induction of TIMP2 with mechanical load may be inducing activation of MMP2, however whether MMP2 is activated and whether it is involved in collagen degradation in response to mechanical loading remains to be determined. ADAMTS1 and ADAMTS5 mRNA were decreased after 24 hours of 3% cyclic strain superimposed on a static 2% load in a study on rat tail tendon fascicles (Maeda, Fleischmann et al. 2010). An ovine tendon model reported decreased ADAMTS4 mRNA in overstrained areas of infraspinatus tendon (due to partial width laceration) (Smith, Sakurai et al. 2008). ADAMTS1, ADAMTS4 and ADAMTS5 all posses some aggrecanase activity, and as they are all decreased in response to mechanical loading this suggests that mechanical stimulation reduces the breakdown of proteoglycan in the ECM, stabilising the tissue. No other studies have reported ADAMTS or TIMP regulation despite their involvement in matrix turnover. This area requires further investigation.

1.7.9. Matrix protein regulation with mechanical strain

Mechanical strain has also been shown to increase collagen type I and III protein and overall collagen content in vivo, ex vivo and in vitro tendon studies (Yang, Crawford et al. 2004; Screen, Shelton et al. 2005; Heinemeier, Olesen et al. 2007; Maeda, Shelton et al. 2009; Szczodry, Zhang et al. 2009). However, a rat study showed that there was no change in collagen type III mRNA with mechanical load (Legerlotz, Schjerling et al. 2007). An increase in type I collagen with mechanical strain indicates that loading induces growth and adaptation of the ECM. Proteoglycan regulation has also been reported in a rat tail tendon model. After a cyclic loading regime of 3% overlaying a 2% static strain for 24 hours decorin, fibronectin and biglycan mRNA was decreased, increased and unchanged respectively (Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010). As decorin and biglycan are involved in the regulation of collagen fibrillogenesis through the interaction of the core protein, which results in the decrease in fibril diameter (Danielson, Baribault et al. 1997; Chakravarti, Magnuson et al. 1998; Svensson, Aszodi et al. 1999), strain modulation of decorin may increase the size of collagen fibrils, increasing tendon strength. An increase in fibronectin in response to mechanical strain may alter cellular interaction with the ECM, as fibronectin is a major glycoprotein involved in the interaction with integrins (Pankov and Yamada 2002).

1.7.10. Cytokine regulation in response to Mechanical load

IL6 protein was increased in humans after prolonged running exercise (36km) (Langberg, Olesen et al. 2002). In addition a bovine study of fatigue cyclic loading (30% elongation at 1Hz) reported an increase in IL6 mRNA and decrease in IL6R mRNA (Legerlotz, Jones et al. 2011). Immunohistochemical analysis showed that IGF1 protein was markedly increased with mechanical loading after twelve weeks of treadmill exercise in a rat model (Scott, Cook et al. 2007). However, a rat tendon study of a 3% cyclic strain overlaying a 2% static strain showed no significant change in IL6 or IGF1 (Maeda, Fleischmann et al. 2010). The lack of agreement between these studies may stem from the fact that strain has variable effects on gene expression. Mechanical loading increased the expression of TGFβ at the mRNA and protein level in a number of studies in both rat tendon and human patellar fibroblasts (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004; Heinemeier, Olesen et al. 2007; Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010), however TGFβ mRNA expression did not change with the application of load in a

rat wheel running study (Legerlotz, Schjerling et al. 2007). IL6, IGF1 and TGFβ are cytokines that have the potential to induce downstream signalling pathways in response to their stimulation. Therefore regulation of these cytokines may be involved in the mechanical regulation of downstream effectors.

Studies of rabbit and human patella fibroblast also showed an increase in COX2 (2 fold) with 20h 6MPa static strain (Asundi and Rempel 2008) or after 4 hours of 4% cyclic strain at 0.5Hz (Yang, Im et al. 2005) respectively. A rat tendon study of a 3% cyclic strain overlaying a 2% static strain showed an significant increase in COX2 after 24 hours (Maeda, Fleischmann et al. 2010). COX2 regulation has as yet no clear role in mechanical regulation. Rodent tissue studies have also measured IL1β in response to mechanical load; a rat in vivo model showed that a 1.7% cyclic load caused an increase in IL1β, however a 0.6% cyclic load decreased IL1β (Sun, Li et al. 2008), in rabbit flexor tendon tissue strained at 4MPa for 20 hours IL1β was decreased 2 fold (Asundi and Rempel 2008). This suggests that above a certain threshold of strain IL1β is increased and below this threshold IL1β is decreased. This fits well with the assumption that with high levels of strain there is a catabolic response and with low levels of strain there is an anabolic response, as IL1 can stimulate MMP activity (Nagase and Woessner 1999).

1.7.11. Mechanical regulation of 'markers' of tendon

There are no true markers of tendon phenotype; however, genes that are more commonly expressed in the tendon rudiment include Scleraxis. Scleraxis has been determined as a marker for tendon and ligament progenitor cells during development (Schweitzer, Chyung et al. 2001). Multipotent stem cells that have the potential to differentiate into tenocytes, stimulation with cyclic and static mechanical load induced the expression of scleraxis (Scott, Danielson et al. 2011). Therefore mechanical stimulation may be a key regulator in tenocyte homeostasis and may induce tenocytes to show more rudimentary characteristics.

Taken together this data suggests that moderate straining regimes may therefore maintain the ECM, although there appears to be a threshold above which too much strain can induce degradative effects upon the surrounding matrix. Whilst magnitude of strain has been previously implicated as more of a key regulatory factor (Arnoczky, Tian et al., 2002), in 2003 Arnoczky's group published data to suggest that frequency was also important in the regulation of rat collagenase (Lavagnino, Arnoczky et al. 2003). Duration of loading may also play an important role (Smith, Sakurai et al. 2008). Therefore magnitude, duration and

frequency are important in how cells respond to strain. More intensive study in this area is required to fully understand how these factors differentially regulate gene expression in human tendon.

1.7.12. Mechanical load and tendinopathy

Tendons of different anatomical positions have differences in composition and rate of turnover, for example positional and energy storing tendons; this is thought to be due to their distinct functions and variation in their exposure to mechanical load (Birch 2007). Tenocytes respond differently depending on the type of strain, for example tenocytes exposed to more compressive strains are morphologically similar to chondrocytes in that they are more spherical in nature (Perez-Castro and Vogel 1999). In addition, these tissue regions are more fibrocartilagenous with increased expression of type II collagen and aggrecan (Perez-Castro and Vogel 1999). Tendons not exposed to high levels of stress, such as the biceps brachii tendon, do not undergo such extensive matrix renewal and in these tendons only very low expression of MMP1, MMP2 and MMP3 is observed. As this type of tendon is less commonly affected by tendinopathy we may conclude that stress may be an important factor in tendinopathy development (Riley, Curry et al. 2002).

Despite this link between tendinopathy and mechanical loading, a level of tension is required to maintain healthy tendon as stress deprivation causes increased ECM breakdown (Arnoczky, Lavagnino et al. 2008; Egerbacher, Arnoczky et al. 2008). And although this association between tendinopathies and increased mechanical strain still remains, there is speculation as to whether tendinopathies are the result of an increase or absence of mechanical loading (Arnoczky et al, 2002). Mechanical failure (fibril damage) of tendon (possibly due to high levels of strain) may result in areas of stress deprivation and other areas of increased stress. Therefore tendinopathies may occur as a result of abnormally distributed stress rather than simply excessive load (Arnoczky et al, 2002). For example, the stress deprivation of rat tail tendon is known to cause the up-regulation of catabolic mediators such as rat collagenase / MMP13 which degrade the matrix and as a result decrease the strength of the tendon (Lavagnino, Arnoczky et al. 2003; Arnoczky, Lavagnino et al. 2008). Stress deprivation has been shown to induce apoptosis in a rat-tail tendon model (Egerbacher, Arnoczky et al. 2008). It has been theorized that apoptosis can occur due to the loss of cellular homeostatic tension caused by fibril damage in vivo (Arnoczky, Lavagnino et al. 2008; Egerbacher, Arnoczky et al. 2008). As physiologically relevant levels and types of strain (<6%) (Lavagnino, Arnoczky et al. 2003; Screen, Lee et

al. 2004) show anabolic changes in MMP mRNA expression. Increases in MMP expression may be due to a relaxation period after strain (Archambault, Tsuzaki et al. 2002; Tsuzaki, Bynum et al. 2003; Sun, Andarawis-Puri et al. 2010) or short term loading (Yang, Im et al. 2005; Maeda, Shelton et al. 2009), this may induce a shift in the homeostasis which in turn increases MMP expression. It is therefore likely that stress deprivation due to localised fibrillar damage plays a key role in the development of tendinopathy.

1.8. MECHANOSENSATION AND TRANSDUCTION

Signal transduction as a result of mechanical stimulation is often referred to as mechanotransduction. Mechanical strain induces the activation of a network of intracellular signalling pathways that are not yet fully understood (Ashida, Takechi et al. 2003; Riddle, Taylor et al. 2006; Craig, Haimovich et al. 2007). Studies of tendon mechanotransduction are limited. However studies covering a wide range of tissues have shown that (1) mechanosensation can involve focal adhesions, integrins, ion channels and growth factors and (2) mechanotransduction can involve calcium, mitogen activated protein kinases (MAPK), Rho/Rho associated protein kinase (ROCK), nuclear factor kappa B (NFκB) and stress activated protein kinases (SAPK) such as c-JUN end terminal kinases (JNK) (Figure 1.5) (For reviews of mechanotransduction see (Banes, Tsuzaki et al. 1995; Lambert, Nusgens et al. 1998; Chiquet 1999; Chiquet, Custaud et al. 2003; Sarasa-Renedo and Chiquet 2005; Wall and Banes 2005; Wang 2006; Wang, Tytell et al. 2009)).

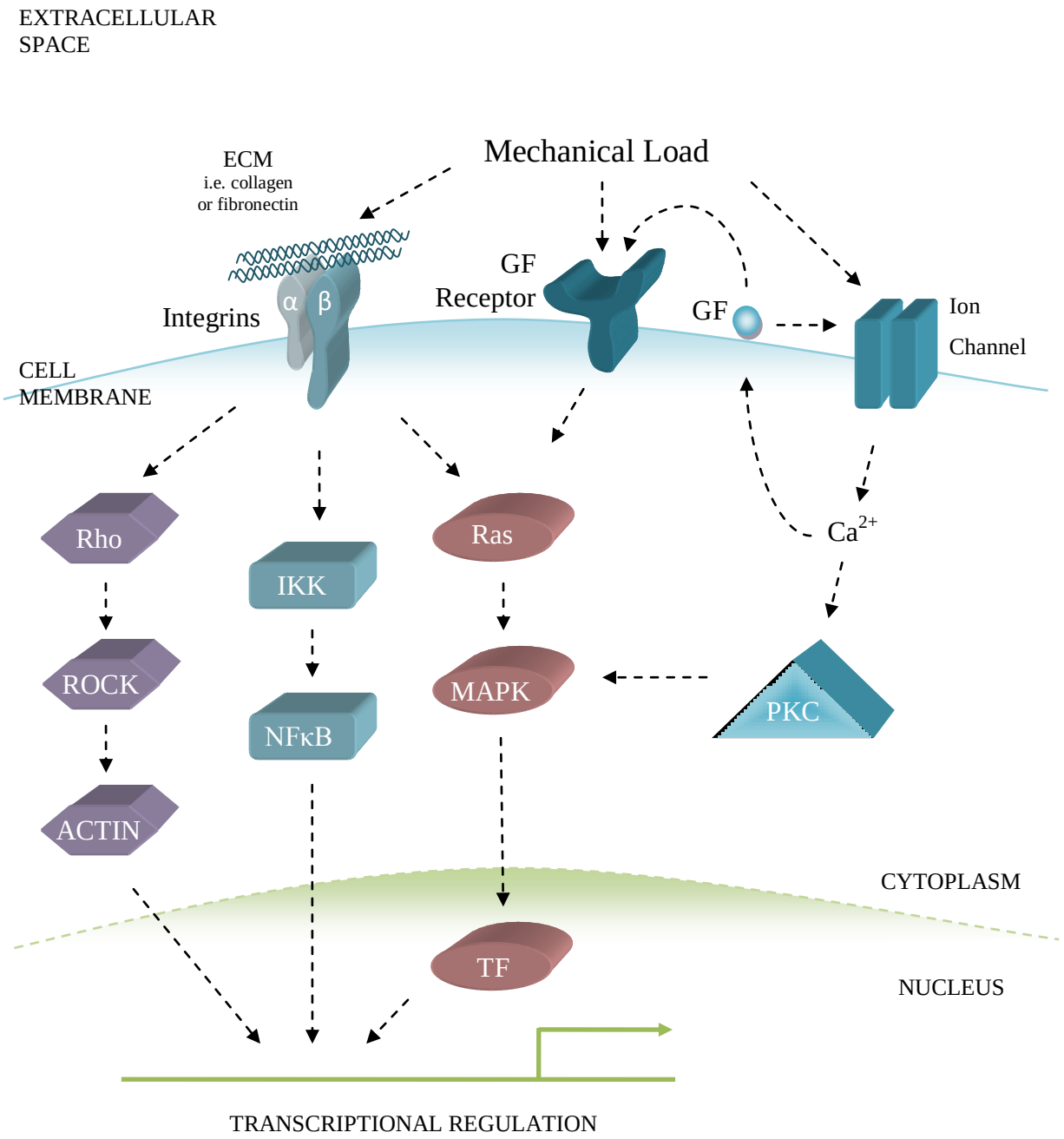

Figure 1.5. Interaction between integrin, growth factor and calcium mediated mechanotransduction. Mechanical load can stimulate the activation of integrins, growth factors and ion channels (i.e. stretch activated ion channels). Consequent intracellular signalling via Rho/ROCK, NFκB and MAPK regulates gene transcriptional regulation. NFκB, Nuclear factor kappa B; IKK, inhibitor of NFκB kinase; MAPK, mitogen activated protein kinase; TF, transcription factor; GF, growth factor; PKC, protein kinase C. Adapted from (Sarasa-Renedo and Chiquet 2005)

1.8.1. Cytoskeletal detection of mechanical strain

The 'mechanostat set point' refers to the level of stress that is required to induce a response to mechanical load (Arnoczky, Lavagnino et al. 2008). A deviation from this set point results in the modification of the ECM either in an anabolic or catabolic fashion. Changes in strain exposure can result in the recalibration of this 'mechanostat set-point' (Frost 1987; Arnoczky, Lavagnino et al. 2008), so that the threshold of strain required to maintain the ECM is modified due to changes in the pericellular environment and resulting modified cell-matrix interaction. For example, stress deprived tendons had an increased MMP13 expression compared to freshly extracted controls. Only by up-regulating this strain (following the period of stress deprivation) did MMP13 expression match that of the loaded controls (Arnoczky, Lavagnino et al. 2008). The idea of resetting of the 'mechanostat set-point' could explain why treatments of eccentric overload are effective. Interventions specifically designed to reset the mechanostat set-point might be effective for tendinopathy treatment (Arnoczky, Lavagnino et al. 2008). Tensegrity and regulation of the 'mechanostat set point' is facilitated by a form of mechanotransduction.

Integrins are transmembrane proteins made up of heterodimers of α and β subunits devoid of any intrinsic tyrosine kinase activity (Hynes 1992). There are 14 α subunits and 8 β subunits which bind non-covalently. Integrin αβ dimers can exist in an open or closed confirmation; this corresponds to their active or inactive nature, respectively (Hynes 1992). When active, integrins function can as an interface between the cell and ECM by binding to components of the ECM. They are also reported to induce signal transfer (Hynes 1992). The cytoplasmic tails of some integrins are linked to the cytoskeleton, the major structural components of the cell. This allows the cytoskeleton to form a direct link with the ECM, sensitising the cell to changes in mechanical load. Integrins form clusters known as focal adhesions; at this site the cytoplasmic domain is connected to a range of adaptor proteins including vinculin, tensin, paxillin, α-actinin and talin. Other proteins such as kinases co-localise with the adaptor proteins; these include focal adhesion kinase (FAK) or integrin linked kinase (ILK) (Banes, Tsuzaki et al. 1995; Ingber 1997; Sarasa-Renedo and Chiquet 2005). The application of mechanical strain to the integrin-ECM ligand complex can induce an increase in the formation of focal contacts and integrin activation. As a result of load-induced integrin activation, the cytoplasmic tail of the β chain can induce tyrosine phosphorylation and consequent activation of FAK. The phosphorylation of adaptor

proteins and tyrosine kinases such as Src can trigger the activation of downstream signalling pathways including MAPK, NFκB and Rho/Rock signalling (Lambert, Nusgens et al. 1998; Sarasa-Renedo and Chiquet 2005). The consequences of these signalling cascades often involve the control of gene expression. Therefore the interaction between integrins and the cytoskeleton forms a mechanical load sensor.

Wang and colleagues suggest that mechanical stimulation can be detected by the direct linkage of the focal adhesions to the nucleus via cytoskeletal components and associated proteins. This association is reported to modulate transcription by chromatin recombination and DNA melting via spatial manipulation, or induce nuclear transport by increasing the size of nuclear pores through nuclear distortion (Wang, Tytell et al. 2009). However, this signalling response has not been confirmed in tendon.

In vitro tendon studies have shown that strain alters tenocyte ECM conformation which in turn alters the shape of the cell and the actin cytoskeleton (Lavagnino, Arnoczky et al. 2003). Tendon tissue exposed to physiological strain regimes shows cellular and nuclear deformation in the direction of the applied force (Arnoczky, Lavagnino et al. 2002). And although local strains perceived by the cell are much smaller than those applied to the whole tendon in the physiological environment, it is possible that the deformation of the cell may invoke a mechanotransduction response (Arnoczky, Lavagnino et al. 2002). Actin filament depolarization causes the abrogation of strain induced MMP1 inhibition, suggesting that the actin cytoskeleton is involved in MMP1 regulation (Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004). Therefore actin filaments may mediate mechanosensation in tendon.

Numerous studies have looked at the response of other cell types to mechanical loading. These include epithelial, endothelial, bone and muscle derived cells (Katsumi, Naoe et al. 2005; Ali, Mungai et al. 2006; Brancaccio, Hirsch et al. 2006). An example of mechanical loading in another tissue is the exposure of cardiomyocytes to stress in response to hemodynamic overload. Integrin isoform β1D is involved in the detection of mechanical load in these cells. Integrin associated proteins including FAK and Src kinases are activated and consequently trigger downstream signalling pathways that include AKT, RAS and ERK (Brancaccio, Hirsch et al. 2006). In a fibroblastic cell line mechanical load induced the activation of αvβ3 integrins via PI3K/Akt signalling. Activation of integrins increases their affinity to bind to the extracellular matrix, creating more adhesion sites and

activates JNK as a downstream signalling pathway (Katsumi, Naoe et al. 2005). Therefore the activation of different signalling pathways in response to strain appears to be cell type specific.

Endothelial cells are exposed to high levels of mechanical stimulation during hypertension (Ali, Mungai et al. 2006). Endothelial cell mechanical stimulation induced the release of reactive oxygen species (ROS) through the displacement of the cytoskeleton and mitochondria (Ali, Mungai et al. 2006). The ROS stimulated the activation of FAK through the protein kinase C (PKC) pathway (Ali, Mungai et al. 2006). This demonstrates that crosstalk exists between signalling pathways in response to mechanical stimuli.

Other signalling pathways have been found to be activated in response to mechanical loading without apparent cytoskeletal or integrin involvement. Tenascin-C is expressed in tissues that are exposed to high levels of strain (Martin, Mehr et al. 2003), including tendon (Riley, Harrall et al. 1996). In response to mechanical load, tenascin-C was induced via the Rho/ROCK and not via ROS, IL4 or MAPK signalling pathways in skin fibroblasts (Chiquet, Sarasa-Renedo et al. 2004). Mechanical load also stimulates NFκB signalling in a magnitude-dependant manner in rabbit articular cartilage (Agarwal, Deschner et al. 2004). At a high cyclic strain (15-18%), nuclear translocation of NFκB was up-regulated; however a low level cyclic strain (4-8%) showed no induction. The stimulation of NFκB in combination with NO (nitric oxide) production could be involved in the initiation of pro-inflammatory events due to excessive loading (Agarwal, Deschner et al. 2004). Therefore mechanical strain can regulate a number of mechanotransduction pathways, with differential activation of these pathways depending upon cell type and the surrounding ECM, as well as the magnitude of mechanical stimulation.

Studies of mechanical loading in tendon have also highlighted signalling pathways in the absence of apparent integrin or cytoskeletal involvement. SAPKs, JNK1 and JNK2 are consistently induced in response to mechanical load in human, canine and rabbit patellar studies (Arnoczky, Tian et al. 2002; Skutek, van Griensven et al. 2003; Kawabata, Katsura et al. 2009). The rabbit tendon studies also showed an increase in phospho-p38 in the absence of mechanical load (Kawabata, Katsura et al. 2009). In the same study markers of apoptosis were also increased. JNK signalling can be associated with apoptosis (Kawabata, Katsura et al. 2009), however not all cyclic loading is associated with increased apoptosis (Kawabata, Katsura et al. 2009). In the studies that showed increased apoptosis, a

prolonged period of mechanical stimulation induced stress tolerance via heat shock protein-mediated suppression of apoptosis (Skutek, van Griensven et al. 2003). In addition, the canine studies showed that induction of JNK was facilitated by calcium signalling (Arnoczky, Tian et al. 2002). This indicates that calcium signalling may play a role in the regulation of downstream signalling pathways in response to tendon loading.

1.8.2. Stress deprivation

Not only is increased mechanical stimulation detected via cellular interaction with the matrix, an absence of tension can also be detected. Studies have shown that by reducing the level of tensional force upon cells in a collagen gel, by gel release, gel contraction and MMP expression is stimulated (Langholz, Rockel et al. 1995; Riikonen, Westermarck et al. 1995; Lambert, Lapiere et al. 1998; Ravanti, Heino et al. 1999). MMPs are of particular interest as increased expression can result in the degradation and potential weakening of the matrix. Studies of this kind have mainly focused on human skin fibroblasts. They have shown that integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are involved in the detection of mechanical stimulus with the involvement of the actin cytoskeleton (Langholz, Rockel et al. 1995; Riikonen, Westermarck et al. 1995; Lambert, Lapiere et al. 1998; Ravanti, Heino et al. 1999). Tyrosine kinase activity, PKC and p38 MAPK signalling was involved in the regulation of the majority of MMP genes (including MMP1, MMP3 and MMP13). Although regulation of MMP9 did not involve p38 MAPK (Lambert, Colige et al. 2001) and ERK signalling had a dampening effect upon MMP13 expression (Ravanti, Heino et al. 1999). MMP2 and MMP14 were not regulated by PKC, MEK1/2 or p38 MAPK (Lambert, Colige et al. 2001), therefore there regulation must occur through another mechanism. MMP gene expression however was not regulated by Rho kinase signalling (Lambert, Colige et al. 2001).

Stress deprivation in osteoblast like cells increased the expression of α2 integrin and MMP3. This regulation was mediated by ROCK and ERK signalling pathways. While contraction of the gels was mediated by ROCK and p38 MAPK and was based on the integrity of microtubules (Parreno and Hart 2009). Another bone related study showed that MMP1 regulation with stress deprivation was mediated via $\alpha_2\beta_1$ integrin, similar to that shown in skin fibroblast studies discussed above (Riikonen, Westermarck et al. 1995). Retinal pigment epithelial cells contracted under stress deprivation, this response was mediated via integrin α_2 and α_3 as well as FAK-Src intracellular signalling pathways (Morales, Mareninov et al. 2007). Therefore the majority of stress deprivation studies have shown that integrin α_2 is involved in the tension perception and that p38 MAPK is the main

signalling molecule involved in the regulation of contraction and MMP expression. However, there are differences in signalling responses between contraction and MMP expression, as well as in different cell types.

Tendon studies of stress deprivation showed an increase in contraction and MMP1, and a decrease in collagen mRNA expression. Once the contraction was abated the level of MMP1 and collagen expression returned to normal levels. This indicates that the cells regulate their own tensional homeostasis. Similar results were also seen in response to cytochalasin treatment where the cytoskeleton is disrupted. This emphasises that the cytoskeleton is a major sensor of cellular tension in tendon cells (Lavagnino and Arnoczky 2005). A recent ex vivo study of lax rat tail tendon fascicles showed similar results. The lack of tension induced an increase in MMP13 expression as well as contraction of the tendon fascicle. Contraction was inhibited by cytochalasin D, indicating the involvement of the actin network. An increase in α-SMA protein expression occurred in parallel with the increase in contraction. Therefore α-SMA may be involved in the contraction, although this has not been confirmed. α-SMA is a marker for myofibroblasts, myofibroblasts are known for their increased contractility (Hinz, Phan et al. 2007); this further indicates that α-SMA may play a role in cell contraction. By pinning these fascicles the induction of MMP13 was abated. Indicating that the increase in MMP13 expression is key in the re-establishment of tensional homeostasis (Gardner, Lavagnino et al. 2012). No other MMPs were measured in response to stress deprivation in these tendon studies and the signalling pathways that regulate this response were not studied further. However, evidence from other cell lineages suggests that integrins, FAK, Src and MAPK may be involved.

1.8.3. Growth factors

Growth factors are regulated in response to mechanical strain. For example TGFβ is sequestered in the ECM as an inactive complex (Taipale, Saharinen et al. 1996; Nunes, Gleizes et al. 1997; Verderio, Gaudry et al. 1999). Upon mechanical stimulation, integrin interaction with the inactive complex induces the activation of TGFβ through tensional forces (Munger, Huang et al. 1999). In addition, TGFβ is activated in response to mechanical loading in a thrombospondin-mediated mechanism in platelet-derived TGFβ (Ahamed, Janczak et al. 2009).

Mechanical load-mediated induction of growth factors also extends to the stimulation of IL1β. In bone, mechanical stimulation above a certain threshold induced IL1β through

integrin and tyrosine kinase activation (Salter, Wallace et al. 2000). IL1β stimulated the depolarisation of calcium activated potassium channels through phospholipase C (PLC), inositol (1,4,5)-triphosphate (IP_3), PKC and prostaglandins (Salter, Wallace et al. 2000). This highlights the complex interaction of kinase signalling with growth factor stimulation. These interactions may have their place in tenocyte signalling.

1.8.4. Gap Junction communication

Gap junctions are transmembrane channels that connect the cytoplasm of adjacent cells. The gap junction is formed from hemichannels located within the cell membrane of adjacent cells. Hemichannels are formed from hexameric oligomer transmembrane proteins, known as the connexins (Pitts, Kam et al. 1987; Kar, Batra et al. 2012). Gap junctions allow small ions to pass freely between cells (Pitts, Kam et al. 1987) as well as low molecular mass molecules such as calcium, IP_3 and cyclic adenosine monophosphate (cAMP) (Figure 2)(Saez, Berthoud et al. 2003). Hemichannels are thought to be involved in the passage of Na^+ (Sodium), Ca^{2+}, glutamate, ATP, PGE_2 (prostaglandin E_2) and NAD^+ in and out of the cell (Kar, Batra et al. 2012). IP_3 is involved in the release of calcium from intracellular stores; therefore passage of IP_3 from one cell can propagate a calcium signal in the adjacent cell (Boitano, Dirksen et al. 1992). Rat flexor tendons showed differential distribution of connexin molecules on the cell surface (McNeilly, Banes et al. 1996). Cx43 and Cx32 were localized to areas of the epitenon, closely associated with collagen bundles, and generally associated with connections between cell bodies. Cx43 was also associated with cell processes (McNeilly, Banes et al. 1996). Cx43 has been reported to increase at the mRNA level in response to both fluid induced shear stress (1dyne/cm^2 for 1 hour and 18 hours rest) and cyclic loading (3.5% at 1Hz for 8 hours, biaxial in monolayer) in avian tendon cells (Elfervig, Lotano et al. 2001; Wall and Banes 2004).

Egerbacher and colleagues, showed that with stress deprivation there is up-regulation of Connexin43 (mRNA and protein) in the pericellular matrix (Egerbacher, Caballero et al. 2007). These GAP junction components may be up-regulated to compensate for the lack of cellular communication (Egerbacher, Caballero et al. 2007). The regulation in GAP junction connexins with mechanical load is consistent with their role in mechanotransduction. The association of Cx43 with actin is increased with mechanical loading in human and avian tenocytes, the actin cytoskeleton is thought to stabilise gap junctions and therefore maintain the interaction of cells during periods of high level mechanostimulation (Wall, Otey et al. 2007). I have previously mentioned the importance

of the cytoskeleton in integrin mediated mechanosensation; here we discuss the association of gap junctions with the actin cytoskeleton. Therefore the cytoskeleton clearly plays a key role in mechanosensation and transduction.

TGFβ has been implicated in the control of Cx43 expression via a non SMAD (c-jun/AP-1, p38 and PI3K/AKT) mediated pathway in mammary gland epithelial cells (Tacheau, Fontaine et al. 2008). In addition, in cardiomyocyte studies, Cx43 is involved in the regulation of TGFβ via competitive inhibition of SMAD2/3 binding to microtubules, resulting in the accumulation of SMAD2/3 in the nucleus (through SMAD2 phosphorylation) and therefore induce the activation of target gene transcription (Dai, Nakagami et al. 2007). Mechanical strain has also been shown to regulate TGFβ at the RNA and protein level (Riser, Cortes et al. 1996) as well as connexin expression (Elfervig, Lotano et al. 2001; Wall and Banes 2004). Therefore an intricate mechanical signalling network may exist through strain regulation of TGFβ and connexins, as well as the interaction between TGFβ and connexins and vice versa.

1.8.5. Calcium signalling

Calcium signalling has been reported in response to compression, vibration and fluid flow in various tissues (Wall and Banes 2005). However, few studies have shown changes in calcium as a result of tensional forces in tendon (Wall and Banes 2005). Human studies of cells from flexor digitorum profundus tendon have shown that both fluid flow (1 or 5 dyne/cm^2) (Elfervig, Francke et al. 2000; Franke, Banes et al. 2000) and static elongation (4-6%) (Elfervig 2001; Wall and Banes 2005) cause an increase in intracellular calcium. Avian cell studies of the same tendon showed no effect of 5% cyclic loading (Wall and Banes 2004) or up to 20 dynes/cm^2 (Elfervig, Lotano et al. 2001). A rabbit Achilles tendon study showed an induction of calcium transients in response to indentation with a micropipette. However, in the same study, fluid flow (up to 25 dyne/cm^2) did not induce significant calcium transients. This suggests that the cells had the potential to induce calcium transients but fluid flow was not a sufficient stimulus (Archambault, Elfervig-Wall et al. 2002). A canine ligament study showed fluid flow (25dynes/cm^2) induced significant calcium transients (Hung, Allen et al. 1997). Therefore, the effects appear to be species specific, as human and canine but not rodent or avian studies showed calcium transients in response to mechanical stimulus.

Banes and colleagues have shown that a certain level of extracellular calcium is required to detect fluid flow (Franke, Banes et al. 2000; Wall and Banes 2005). This indicates that extracellular calcium or other molecules such as IP_3 may enter the cell via gap junctions or other calcium channels; i.e. from an extracellular source rather than from intracellular stores. Studies have compared mechanisms of intracellular calcium release in response to 6% static stretch (equibiaxial) and shear stress (2 minutes at 1 dyne/cm^2) in human tenocytes (Elfervig 2001; Wall and Banes 2005). They showed that stretch activated channels were involved in the response to cyclic strain. However in response to shear/fluid flow, stretch activated ion channels and voltage dependant L-type calcium channels were not involved in the increase in intracellular calcium. Multiple other pathways were implicated in the response to shear, these included; partial involvement of cyclooxygenase activation; the involvement of G coupled receptors; and cAMP as a signal modifier (Elfervig, Francke et al. 2000). The requirement of G protein coupled receptors indicates that purinoceptors (just one of the receptor types associated with G proteins) may be involved in fluid shear induced calcium transients from intracellular stores. Purinoceptors induce release of calcium from intracellular stores through the activation of a signalling cascade that involves the activation of a G protein (Discussed below) (Figure 2). Fluid flow also induced calcium transients in canine ligaments cells, this was facilitated by calcium release from intracellular stores, consistent with tendon studies (Hung, Allen et al. 1997). Therefore mechanically stimulated signalling mechanisms appear to be specific to types of mechanical deformation.

In conditions of fluid flow anterior cruciate ligament (ACL) cells were responsive to extracellular calcium, whereas cells derived from the medial collateral ligament (MCL) were not (Hung, Allen et al. 1997). MCL cells were less sensitive to fluid flow, as they have a very robust cytoskeleton this could make them less mechanosensitive (Hung, Allen et al. 1997). Therefore cells derived from different locations as well as different tissues may respond to strain via different mechanisms.

As we have discussed reports indicate that calcium signalling is activated in response to mechanical loading, however, as far as we are aware there is no direct evidence that the resulting changes include regulation of metalloproteinase expression in tendon. Although collagen synthesis as well as DNA synthesis via mechanical loading is inhibited by blockade of calcium signalling via GAP junctions, i.e. the increase in collagen with load was inhibited by the addition of octanol (Gap junction blocker) (Banes, Weinhold et al.

1999; Sood, Bynum et al. 1999). Therefore regulation of collagen and overall DNA synthesis is mediated by GAP junction communication. However, no direct evidence implicates calcium signalling in MMP regulation with strain. In fact Archambault and colleagues showed that MMP1 and MMP3 were increased in response to 1dyne/cm^2 fluid flow without significant calcium transients in rabbit Achilles tendon (Archambault, Elfervig-Wall et al. 2002). However this is a response to fluid flow, a different type of load (i.e. tension) or perhaps human derived cells may induce calcium transients. However this remains to be determined.

Evidence of a complex calcium signalling pathway has emerged through studies of cells from a wide range of tissues. Increased calcium influx can occur not only via GAP junctions but also voltage gated, receptor gated and stretch activated (mechanosensitive) channels. Calcium can also be released from intracellular stores; through calcium binding to ryanodine sensitive receptors in the endoplasmic reticulum (ER); or through ATP binding to purinoceptors (Figure 1.6) (Wall and Banes 2005). ATP is reported to increase as a result of mechanical loading (Graff, Lazarowski et al. 2000; Jones, Wall et al. 2005). It is released from stress activated channels (Naruse, Sai et al. 1998; Naruse, Yamada et al. 1998) as well as connexin hemichannels (Stout, Costantin et al. 2002). ATP, as well as ADP and adenosine can act as activators of purinoceptors. Purinoceptors are involved in multiple physiological functions including inflammation, peripheral and central neuronal transmission and muscle contraction. Purinoceptors are categorised into two main groups, those which respond to ATP or its analogs (ADP, AMP or GTP) and those which are activated by adenosine these are termed P2 and P1 purinoceptors respectively. P2 purinoceptors are further categorised into groups P2X and P2Y; P2X are ligand gated ion channels which are selectively permeable to cations such as calcium, whereas P2Y are G-protein coupled receptors (Valera, Hussy et al. 1994; North 2002; Gever, Cockayne et al. 2006). Activation of P2X purinoceptors (ligand gated ion channels) can cause the influx of cations such as calcium (Gever, Cockayne et al. 2006). ATP acts in an autocrine or paracrine fashion to activate P2Y purinoceptors which results in G protein activation, interaction with phospholipase C, cleavage of phophatidylinositol 4,5-bisphosphate (PIP_2) to produce IP_3 which acts on channels in the ER to release intracellular calcium (Figure 2) (Wall and Banes 2005).

P2Y1 and P2Y2 purinoceptors are responsible for downstream ATP signalling events (increase in intracellular calcium) in rodent Achilles tendon (Fox, Jones et al. 2005; Jones,

Yang et al. 2005). A study of human cartilage showed that compression induced cellular hyperpolarisation and that inhibition of P2Y purinoceptors by Suramin and PPADs abrogated this response (Millward-Sadler, Wright et al. 2004). P2Y purinoceptors are also reported to be involved in increased calcium mobilisation in response to fluid flow in osteoblasts. Further characterisation of this pathway indicated that ATP activated this receptor and caused calcium mobilisation via the inositol 1,4,5-trisphosphate pathway (You, Jacobs et al. 2002). Although it seems that the majority of mechanically stimulated purinoceptors are P2Y forms, stimulation of P2X purinoceptors has also been reported. In endothelial cells a fluid flow induced influx of calcium was reported to involve P2X4 purinoceptors, this was confirmed by the inhibition of P2X4 transcription via antisense oligonucleotides (Yamamoto, Korenaga et al. 2000). Mechanical stimulation of smooth muscle cells induced calcium transients via adenosine and ATP purinoceptor signalling; through P1 and P2 receptors respectively. They also showed that purinoceptor signalling stimulates JNK/SAPK, which results in gene transcriptional modification (Hamada, Takuwa et al. 1998). Although this connection between purinoceptor signalling and JNK has not been shown in tendon, it is possible that it may occur; as JNK activation in response to calcium signalling has been shown in tendon studies (Arnoczky, Tian et al. 2002; Skutek, van Griensven et al. 2003; Kawabata, Katsura et al. 2009).

Reports of a feedback loop involving a decrease in ATP levels during mechanical loading may involve the release of ATPase's (ATP degrading enzymes) and consequent degradation of ATP (Graff, Lazarowski et al. 2000; Yegutkin, Bodin et al. 2000; Tsuzaki, Bynum et al. 2005). To complicate matters further ATP can also negatively modulate strain induced MMP regulation, where an up-regulation of MMP1 with loading is reversed with the addition of ATP, through the dual action of autocrine and paracrine ATP signalling (Tsuzaki, Bynum et al. 2003). Prestress can alter the ability of cells to perceive mechanical load and can be measured by the contractile ability of cells. ATP inhibits the contractile ability of bone cells through MAPK and NFκB signalling and therefore reduces the sensitivity of cells to mechanical loading (Qi, Chi et al. 2007; Qi, Chi et al. 2009). Therefore ATP mediated mechanotransduction appears complex in nature and may involve multiple purinoceptor pathways facilitated by degradation products of ATP. However, this complex system is characterised from mostly non-tendon studies and in not fully understood in tendon.

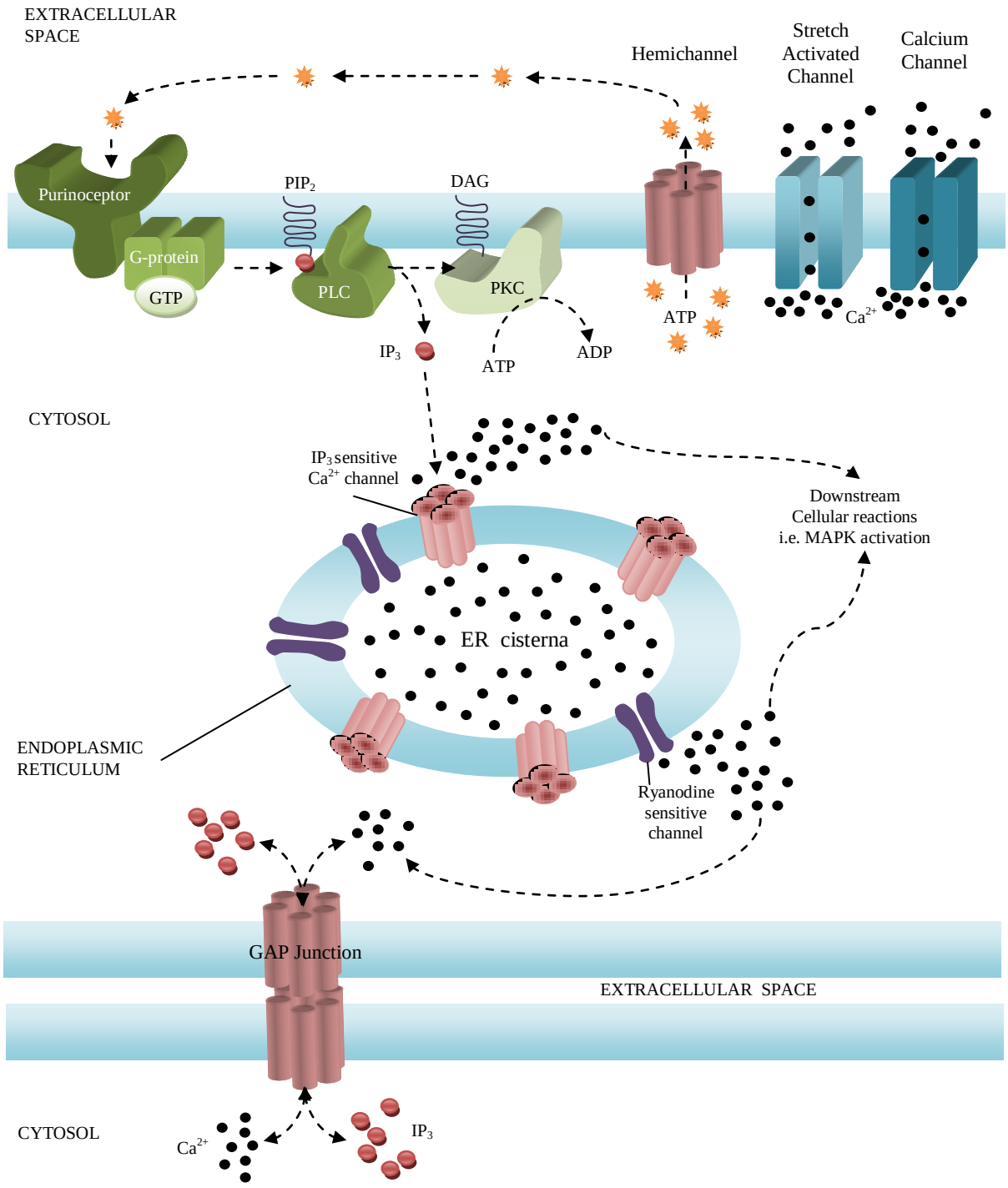

Figure 1.6. Calcium signalling overview. Calcium can enter the cell through stretch activation and calcium ion channels. ATP can be released through hemichannels into the extracellular space and can act in a paracrine or autocrine fashion to activate purinoceptor signalling. ATP, UTP or adenosine binding of purinoceptors induces the activation of G-protein. This involves the replacement of GDP with GTP. Activated G-protein induces an interaction with phospholipase C (PLC) which cleaves phosphatydlinositol 4,5-bisphosphate (PIP_2) into inositol (1,4,5)-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 activates IP_3 sensitive calcium channels on the ER and induces the release of calcium into the cytoplasm. Calcium stimulates downstream signalling events; such as activation of MAPK signalling. DAG induces the activation of protein kinase C (PKC) which is involved in multiple downstream processes. GAP junction communication can involve the transfer of Calcium and IP_3 to adjacent cells. Adapted from (Wall and Banes 2005)

1.9. TRANSFORMING GROWTH FACTOR BETA

Transforming growth factor β (TGFβ) is a cytokine involved in a plethora of cell processes including cell proliferation, differentiation, migration, adhesion and apoptosis (Shi and Massague 2003). TGFβ is known to stimulate deposition and inhibit the degradation of the ECM; through increased collagen (Heinemeier, Olesen et al. 2009) and decreased collagenase and stromelysin expression (Zeng, McCue et al. 1996; White, Mitchell et al. 2000). TGFβ is involved in fibrosis and wound healing and is known to play a role in an array of disease phenotypes including cancer, infectious disease and autoimmune disorders (Worthington, Klementowicz et al. 2011).

The TGFβ superfamily of proteins includes TGFβ, inhibins / activins, bone morphogenic proteins (BMP) and growth and differentiation factors (GDF). In mammals TGFβ exists in 3 isoforms; TGFβ1, TGFβ2 and TGFβ3, each is thought to have a distinct function in tendon, however this is not fully understood (Fenwick, Curry et al. 2001). Knockout mice of each of the three isoforms confirm that each has a distinct function due to the differential effects upon development, including defective haematopoiesis and vasculogenesis in TGFβ1 null mice (*tgf*b1$^{-/-}$) (Dickson, Martin et al. 1995), disruption of epithelial-mesenchymal interactions as well as perinatal mortality in TGFβ2 null mice (*tgf*b2$^{-/-}$) (Sanford, Ormsby et al. 1997) and finally cleft palate and delayed pulmonary development in TGFβ3 null mice (*tgfb*3$^{-/-}$) (Kaartinen, Voncken et al. 1995). TGFβ1, the predominant isoform involved in wound healing, was below the level of detection in both embryonic and adult tendon studies (Fenwick, Curry et al. 2001; Kuo, Petersen et al. 2008). TGFβ2 was found to be the predominant isoform expressed in both normal and pathological human Achilles tendon (Fenwick, Curry et al. 2001).

1.9.1. TGFβ Synthesis and secretion

Newly synthesised TGFβ protein contains a propeptide known as the latency associated peptide (LAP). In the trans-golgi network, post-translational modifications involve cleavage of LAP by proprotein convertase furin (Dubois, Blanchette et al. 2001), TGFβ and LAP remain non-covalently bound once the complex is released into the extracellular space, rendering TGFβ inactive (Gentry and Nash 1990); however the process of inhibition is not yet fully understood. TGFβ plus LAP is known as the small latent complex (SLC). Latent TGFβ binding proteins (LTBP) form cysteine disulphide linkages with TGFβ homodimers via LAP to form the Large Latent Complex (LLC) this occurs before TGFβ is released into the extracellular space (Gleizes, Beavis et al. 1996; Saharinen, Taipale et al. 1996). LTBP is expressed as four variants (LTBP-1, -2, -3 and -4) and is important in the secretion, correct folding (Miyazono, Olofsson et al. 1991) and targeting of LLC to the extracellular matrix where transglutaminases covalently link LTBP to ECM proteins such as fibronectin (Taipale, Saharinen et al. 1996; Nunes, Gleizes et al. 1997; Verderio, Gaudry et al. 1999) (Figure 1). Fibrillin microfibrils stabilise the interaction between LTBP and the ECM, this interaction is assembled pericellularly and the incorporation of fibrillin into this complex involves heparin sulphate (Massam-Wu, Chiu et al. 2010). In the absence of the LTBP, the TGFβ SLC is secreted, however this process is a great deal slower (Miyazono, Olofsson et al. 1991).

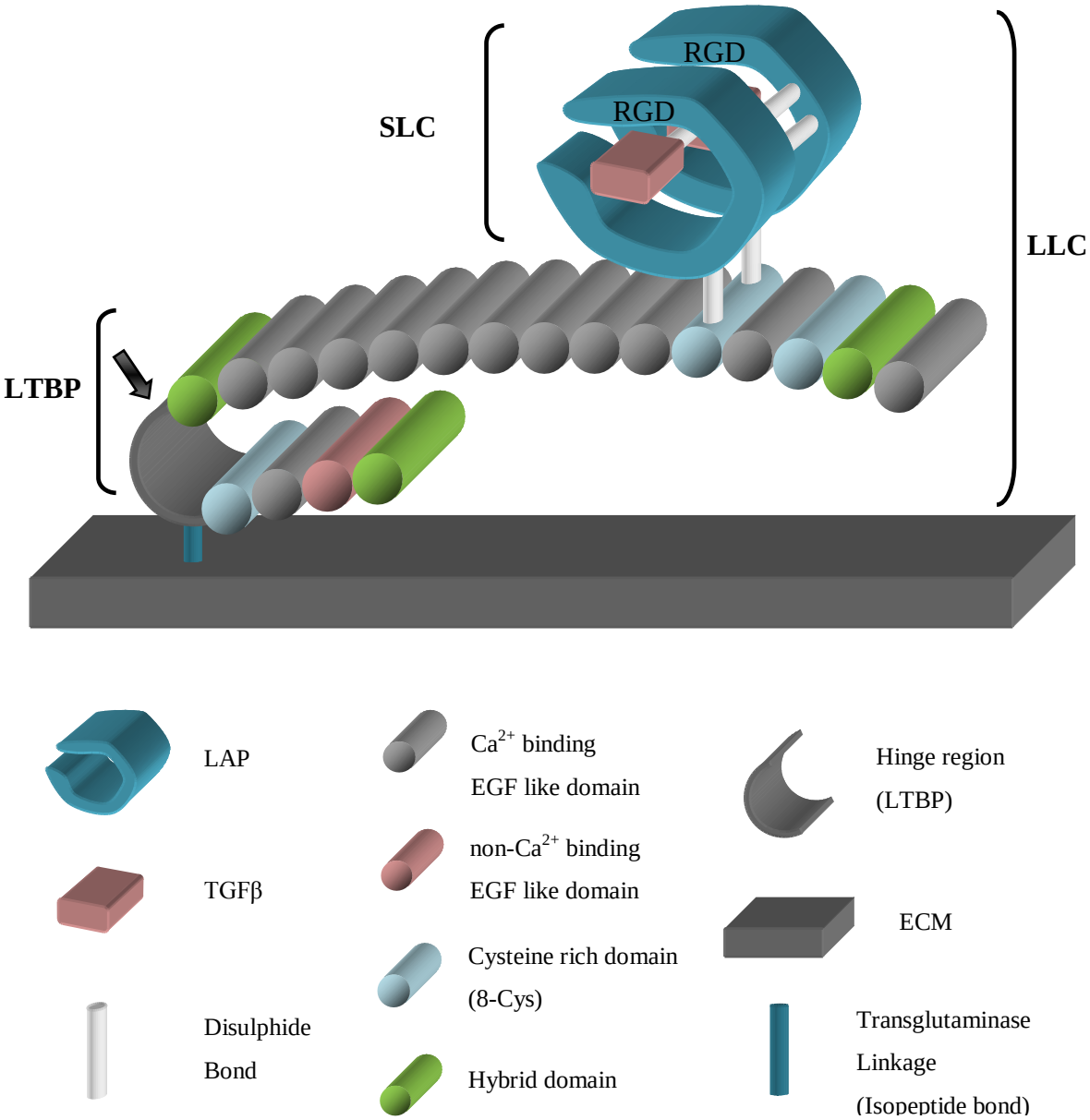

Figure 1.7. TGFβ is deposited in the matrix as a large latent complex (LLC). The LLC is made up of the small latent complex (SLC) and the latent TGFβ binding protein (LTBP) adjoined by disulphide bonds. The SLC consists of TGFβ and its latency associated peptide (LAP) connected non-covalently (due to intracellular processing via furin), this accumulates as a homodimer. LTBP binds to SLC intracellularly and targets the LLC to the ECM. The LTBP is linked to the ECM via its hinge region by isopeptide binding which is facilitated by transglutaminases. LTBP-1 is the most characterised LTBP; it contains 17 EGF like domains, 3 cysteine rich domains and a hybrid domain. The hinge region of LTBP is susceptible to proteolytic cleavage which results in the release of a processed form of the LLC (indicated with an arrow). Adapted from (Annes, Munger et al. 2003; Wipff and Hinz 2008; Worthington, Klementowicz et al. 2011)

1.9.2. Activation of latent TGFβ

Protease mediated activation

In the ECM, as part of the LLC, TGFβ is a convenient store of potentially active TGFβ. Activation has been reported to occur by a variety of mechanisms that involve proteolytic cleavage. Cell surface integrin αvβ8 co-localises with membrane bound protease MT1-MMP, αvβ8 binds with high affinity to LAP via the RGD motif allowing MT1-MMP to proteolytically cleave TGFβ from the complex, rendering TGFβ active and diffusible (Mu, Cambier et al. 2002) (Figure 2).

TGFβ is also thought to be activated by other proteases such as MMP2, MMP3, MMP9, MMP13, thrombin and plasmin (Lyons, Keski-Oja et al. 1988; Taipale, Koli et al. 1992; Yu and Stamenkovic 2000; D'Angelo, Billings et al. 2001; Maeda, Dean et al. 2002). Many of these proteolytic activation mechanisms require cell surface localisation to allow interaction between the latent complex and the active protease. CD44 is a cell surface receptor involved in cell-cell and cell matrix interactions with a principle role in adhesion. CD44 ligands include hyaluronic acid, collagen, fibronectin and laminin (Goodison, Urquidi et al. 1999). CD44 acts as a cell surface docking receptor for both MMP2 and MMP9 which allows the proteolytic cleavage of LAP resulting in the release and consequent activation of TGFβ (Yu and Stamenkovic 2000). αvβ3 has been implicated in autocrine TGFβ signalling which may involve the activation of TGFβ (Asano, Ihn et al. 2005; Wipff, Rifkin et al. 2007), and is reported to co-localise with MMP2 and MMP9. This suggests that metalloproteinase activity may be responsible for the activation of TGFβ in this case, however, this is as yet unconfirmed (Brooks, Stromblad et al. 1996; Rolli, Fransvea et al. 2003) (Figure 3).

Plasmin also cleaves LAP to release active TGFβ1 (Lyons, Keski-Oja et al. 1988; Sato and Rifkin 1989; Lyons, Gentry et al. 1990). Although, once the LLC is sequestered in the ECM, direct activation of TGFβ by plasmin is inhibited, as the plasmin sensitive region of LAP is masked. However plasmin is able to cleave LTBP so that a processed form of the LLC complex is released. This processed form targets Mannose-6-phosphate/Insulin like growth factor type II receptor (M6P/IGF-II) at the cell surface (Taipale, Miyazono et al. 1994; Nunes, Gleizes et al. 1997). Upon localisation M6P/IGF-II can bind to LAP which

facilitates the activation of TGFβ through plasmin mediated proteolytic cleavage (Dennis and Rifkin 1991; Odekon, Blasi et al. 1994; Nunes, Shapiro et al. 1995) (Figure 4).

BMP1 and MMP3 have been reported to degrade LTBP, preventing ECM sequestration of the TGFβ latent complex and increasing levels of diffusible latent TGFβ. However BMP1 is not involved in the secondary protease cleavage and consequent activation of TGFβ, this requires additional protease activity (Maeda, Dean et al. 2002; Ge and Greenspan 2006). MMP3 is also able to cleave LAP and induce the activation of TGFβ once LLC is released (Maeda, Dean et al. 2002). Studies of Geleophysic dysplasia, an inherited condition resembling a lysosomal storage disorder, have shown that ADAMTSL2 (and perhaps ADAMTS10), a gene mutated in the disease, may be involved in the activation of TGFβ through the interaction with LTBP-1, however this process of activation is not fully elucidated (Le Goff, Morice-Picard et al. 2008).

Calpain, a calcium dependant cysteine protease and Cathepsin D, lysosomal aspartyl protease are also reported to activate latent TGFβ (Lyons, Keski-Oja et al. 1988; Abe, Oda et al. 1998). However, Cathepsin activation of TGFβ has only been shown in simple experiments in the absence of cells and calpain activation does not occur in response to the protease alone; therefore these mechanisms may also not be physiologically relevant (Lyons, Keski-Oja et al. 1988; Abe, Oda et al. 1998).

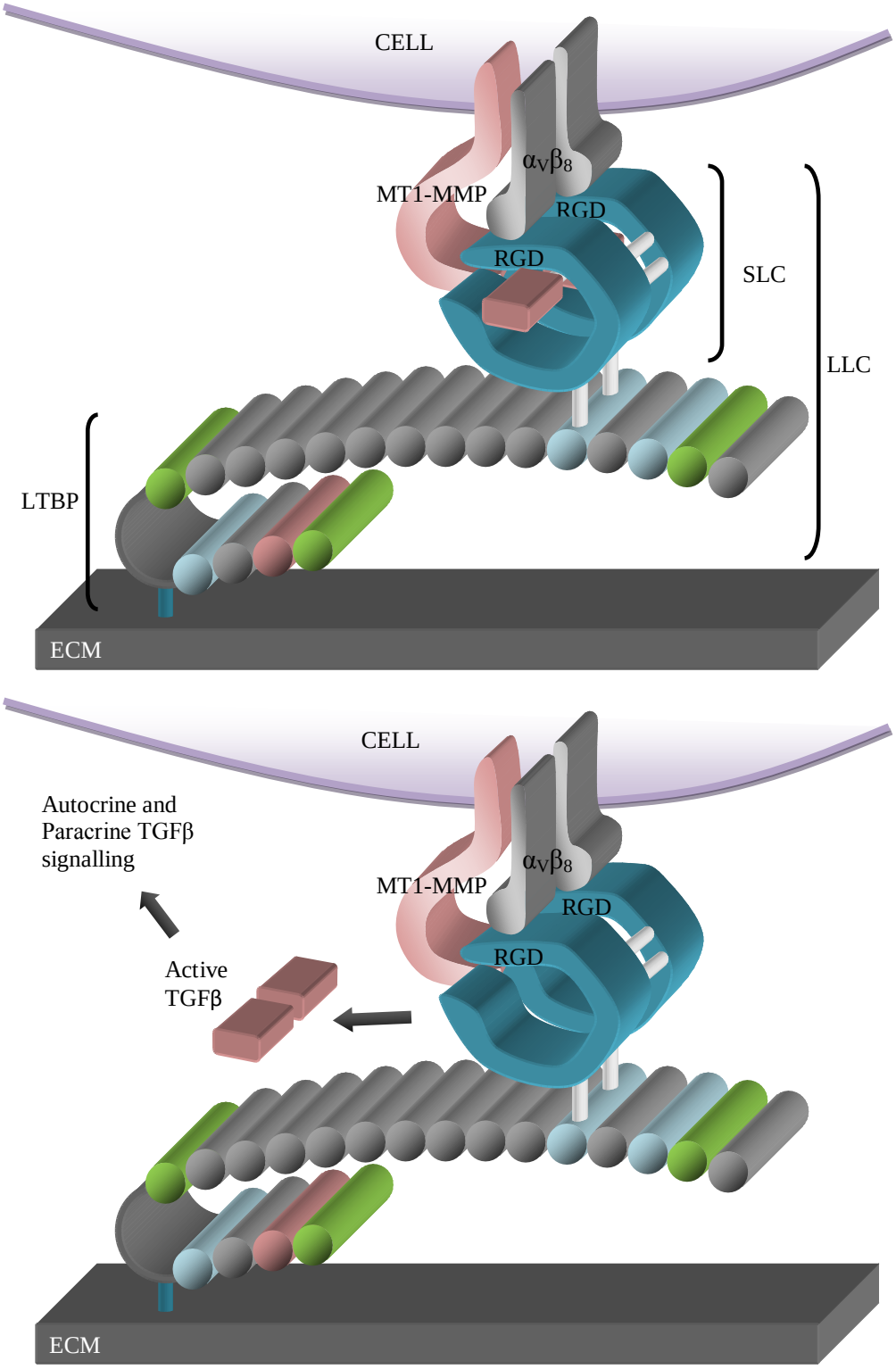

Figure 1.8. αvβ8 and MT1-MMP mediated activation of TGFβ. $\alpha_v\beta_8$ integrins expressed on the cell surface co-localise with membrane bound metalloproteinase MT1-MMP. $\alpha_v\beta_8$ binds to the RGD motif located on LAP. This allows MT1-MMP to cleave active TGFβ from the LLC rendering it active and diffusible. Therefore both autocrine and paracrine signalling can occur. Adapted from (Wipff and Hinz 2008; Worthington, Klementowicz et al. 2011)

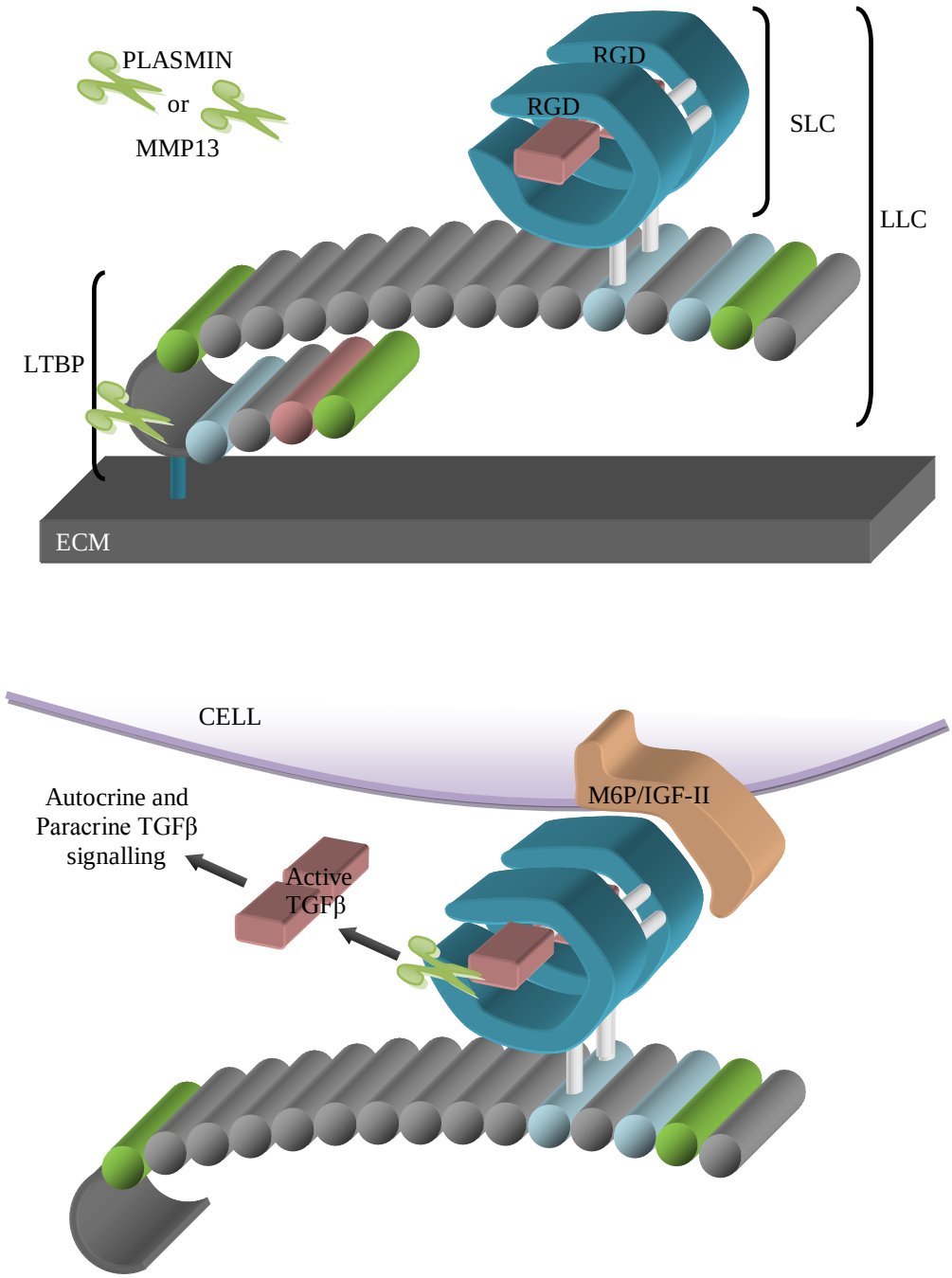

Figure 1.9. Plasmin and M6P/IGF-II mediated activation of TGFβ. Plasmin is unable to access the region of LAP in order to cause activation when the LLC is sequestered in the ECM. Plasmin cleaves the LTBP from the extracellular matrix via the hinge region, this releases a processed form of the LLC complex. LAP is then able to associate with M6P/IGF-11 at the cell surface which allows plasmin to cleave LAP and TGFβ becomes active and diffusible. Diffusible TGFβ is able to induce both autocrine and paracrine TGFβ signalling. Adapted from information gleaned from the following sources (Lyons, Keski-Oja et al. 1988; Sato and Rifkin 1989; Lyons, Gentry et al. 1990; Dennis and Rifkin 1991; Odekon, Blasi et al. 1994; Taipale, Miyazono et al. 1994; Nunes, Shapiro et al. 1995; Nunes, Gleizes et al.

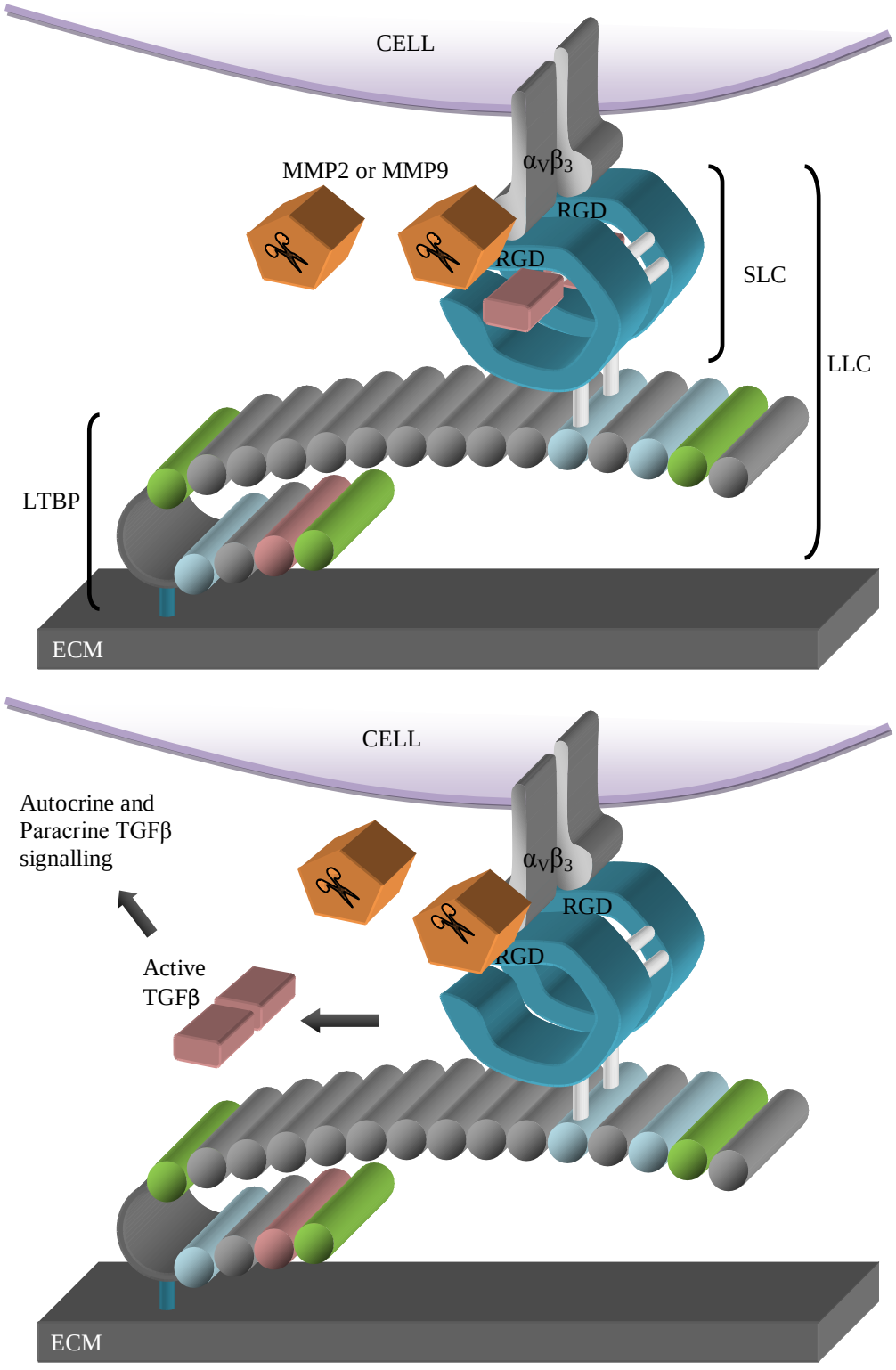

Figure 1.10. αvβ3 and MMP mediated activation of TGFβ. $\alpha_v\beta_3$ integrins expressed on the cell surface co-localise with diffusible MMP2 or MMP9. $\alpha_v\beta_3$ binds to the RGD motif located on LAP. This allows MMP2 or MMP9 to cleave active TGFβ from the LLC rendering it active and diffusible. Therefore both autocrine and paracrine signalling can occur. Adapted from (Wipff and Hinz 2008; Worthington, Klementowicz et al. 2011)

Tensional force mediated activation

Integrins are also involved in the activation of TGFβ in the absence of proteolytic activity. Interaction of integrin αvβ6 with LAP (via an RGD motif) can result in TGFβ activation via a conformational change in the latent complex (Figure 5). This mechanism of TGFβ activation is not fully understood, yet there is evidence to suggest that it involves tensional forces between the cell and ECM, sequestration of the LLC in the ECM via the LTBP, the clustering of integrins and linkage to the cytoskeleton via focal adhesion kinase and paxillin adaptor proteins (Munger, Huang et al. 1999; Annes, Chen et al. 2004). αvβ6 expression however is limited to epithelial cells and direct cell to cell contact is required for activation to occur (Sheppard 2005). αvβ5 also contains an RGD binding motif and is involved in the activation of TGFβ in the absence of protease activity and requires substrate tension as well as an intact cytoskeleton (Asano, Ihn et al. 2005; Wipff, Rifkin et al. 2007). Inhibition of cell contraction with compounds such as blebbistatin and seeding cells on flexible substrates decreases αvβ5 mediated TGFβ activation, demonstrating that the contractile ability of cells also regulates TGFβ activation (Wipff, Rifkin et al. 2007). This indicates tensional forces play a major role in the activation of TGFβ by αvβ5 and αvβ6, with the opposing forces between the ECM, LLC and the cytoskeleton, however exactly how this occurs requires clarification (Wipff, Rifkin et al. 2007).

Other integrins, containing the RGD motif capable of binding to TGFβ LAP are α8β1 and αvβ1; however their involvement in TGFβ activation is not well characterised (Munger, Harpel et al. 1998; Lu, Munger et al. 2002; Sheppard 2005; Worthington, Klementowicz et al. 2011). An unidentified β1 integrin also plays a role in TGFβ activation, however the mechanism to which this occurs is not fully understood (Wipff, Rifkin et al. 2007). In addition to the positive interaction of integrins and activation of TGFβ, blocking α5 integrin stimulates TGFβ production, this suggests that α5 integrin may also be involved in the inhibition of TGFβ protein synthesis (Matsumoto, Ishimura et al. 2003).

αvβ6 has been reported to be involved in the activation of both TGFβ1 and TGFβ3, but not TGFβ2. The LAP of TGFβ2 lacks an RGD motif and therefore is not able to bind integrins as do TGFβ1 or TGFβ3 (Munger, Huang et al. 1999; Annes, Rifkin et al. 2002; Sheppard 2005). As TGFβ2 is not activated via RGD interaction of integrins it must be regulated by

an alternate mechanism, this may not be completely devoid of integrin interaction. However there are other mechanisms of TGFβ activation that do not involve integrins.

TGFβ can also be activated in response to high temperatures (80°C); however this is not physiologically relevant. Activation can also occur with mildly acidic pH (4.5), although this induces only 20-30% activation and is also unlikely to be physiologically relevant (Lyons, Keski-Oja et al. 1988). Reactive oxygen species (ROS) induce the oxidation of specific amino acids of latent TGFβ causing a conformational change and subsequent activation (Barcellos-Hoff, Derynck et al. 1994; Barcellos-Hoff and Dix 1996). However ROS activation of TGFβ appears to be less common than those mechanisms involving proteases and integrins.

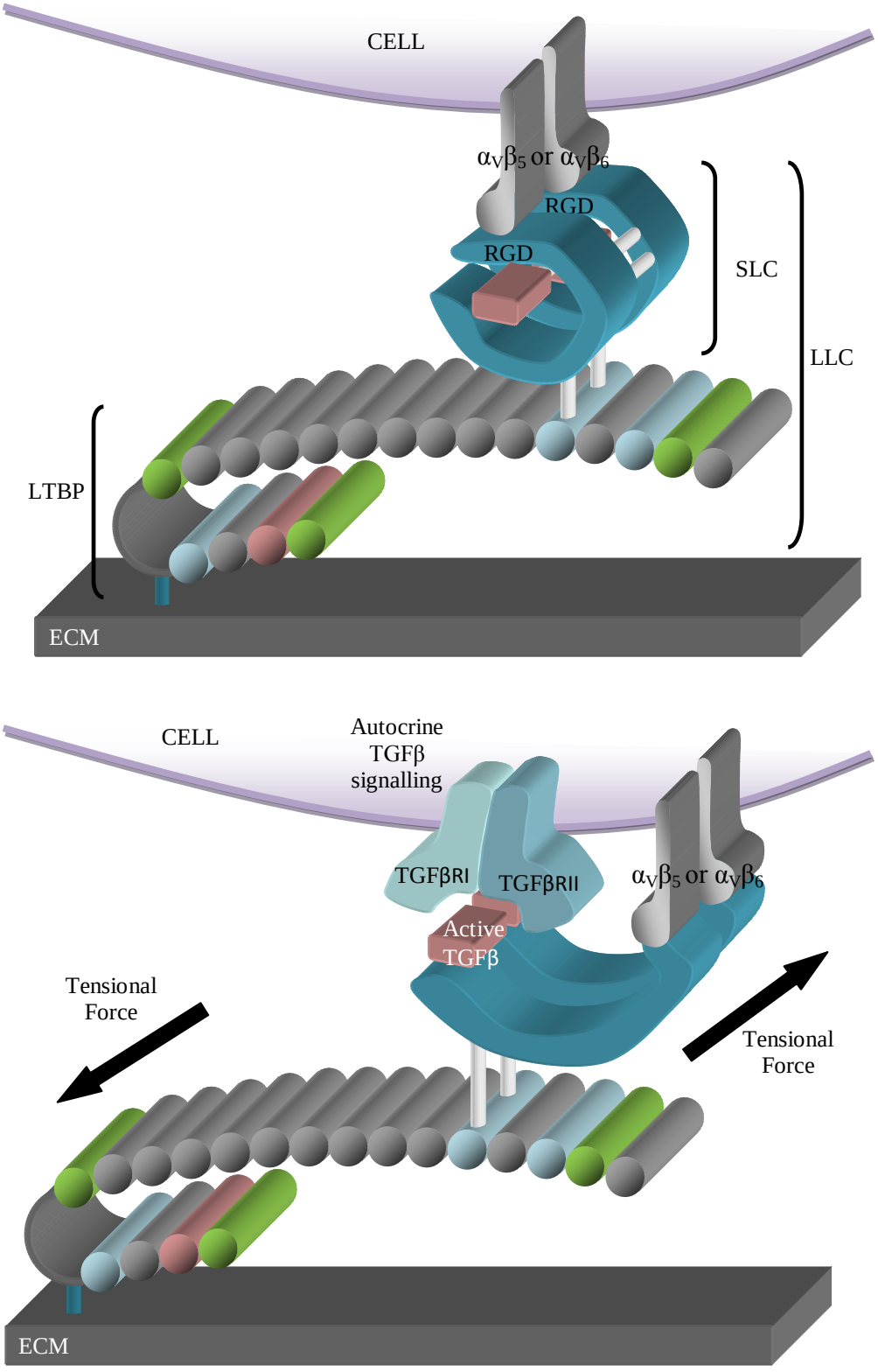

Figure 1.11. αvβ5 and αvβ6 mediated activation of TGFβ via tensional forces. $\alpha_v\beta_5$ or $\alpha_v\beta_6$ integrins expressed on the cell surface bind to the RGD motif located on LAP. As the LLC is sequestered in the matrix and the integrins are linked to the cytoskeleton, tensional forces between the cell and the cytoskeleton increase the distance between the ECM and the cell surface causing a distortion in the LLC resulting in the exposure of active TGFβ. TGFβ can therefore bind to its receptors. TGFβ is not released as a result of this interaction and remains attached to the LLC, therefore only autocrine signalling can occur. Adapted from (Annes, Munger et al. 2003; Wipff and Hinz 2008; Worthington, Klementowicz et al. 2011)

Thrombospondin-1 mediated activation

In the absence of proteolytic activity, certain TGFβ activation mechanisms result in a conformational change in TGFβ rendering it active without detachment from the latent complex, i.e. non diffusible active TGFβ is produced. We have previously mentioned $\alpha_v\beta_5$ and $\alpha_v\beta_6$ integrins acting in this way, a similar mechanism involves thrombospondin-1 (TSP-1). TSP-1 can interact with LAP via an LSKL motif resulting in a conformational change in the latent complex and TGFβ activation (Schultz-Cherry and Murphy-Ullrich 1993; Crawford, Stellmach et al. 1998; Ribeiro, Poczatek et al. 1999) (Figure 6). Diffusible TSP-1 can bind to a cell surface receptor known as CD36, this can effectively localise TSP-1 to the cell surface. TSP-1 can bind to latent TGFβ inducing activation, this can occur either through the aforementioned conformational change in the latent TGFβ complex or cleavage of LAP by plasmin; only the latter mechanism releases diffusible TGFβ (Yehualaeshet, O'Connor et al. 1999). Therefore not only diffusible TSP-1 but also membrane bound TSP-1 (via CD36) can regulate TGFβ activation. TGFβ1 and TGFβ2 are activated by TSP-1 however it is not clear whether thrombospondin-1 also activates TGFβ3 (Crawford, Stellmach et al. 1998). TSP-1 also binds to heparin sulphate proteoglycans located at the cell surface (Sun, Mosher et al. 1989), this localisation of TSP-1 may also be involved in the activation of TGFβ, however this has not been confirmed.

Thrombospondin (TSP-1) null mice show some similarities in phenotype to *tgfb1*$^{-/-}$ null mice, however this is only a partial overlap which suggests that other mechanisms are involved in the activation of TGFβ1 (Crawford, Stellmach et al. 1998). Knockout of individual proteases does not induce phenotypic changes comparable with the *tgf*b1-/- phenotype (Annes, Munger et al. 2003). Integrins on the other hand are thought to be the predominant mediators of TGFβ1 activation. Studies have shown that mice with an RGD to an RGE mutation (*tgfb1*$^{RGE/RGE}$) display the major features of mice with the *tgfb1*$^{-/-}$ mutation including widespread organ inflammation and defects in vasculogenesis, despite normal levels of latent TGFβ (Yang, Mu et al. 2007). In addition knockout of α_v integrins (*Itgav*$^{-/-}$) induces a similar phenotype to *tgf*b1$^{-/-}$ (differential vasculogenesis) and *tgf*b3$^{-/-}$ (cleft palate) in mice (Dickson, Martin et al. 1995; Kaartinen, Voncken et al. 1995; Bader, Rayburn et al. 1998; Worthington, Klementowicz et al. 2011). $\alpha_v\beta_6$ and $\alpha_v\beta_8$ knockout mice ($\alpha_v\beta_6^{-/-}$ and $\alpha_v\beta_8^{-/-}$) also show a similar phenotype to tgfβ1$^{-/-}$ and tgfβ3$^{-/-}$ null mice, this

indicates that αvβ6 and αvβ8 integrin mediated activation of TGFβ1 and TGFβ3 is predominant *in vivo* (Aluwihare, Mu et al. 2009).

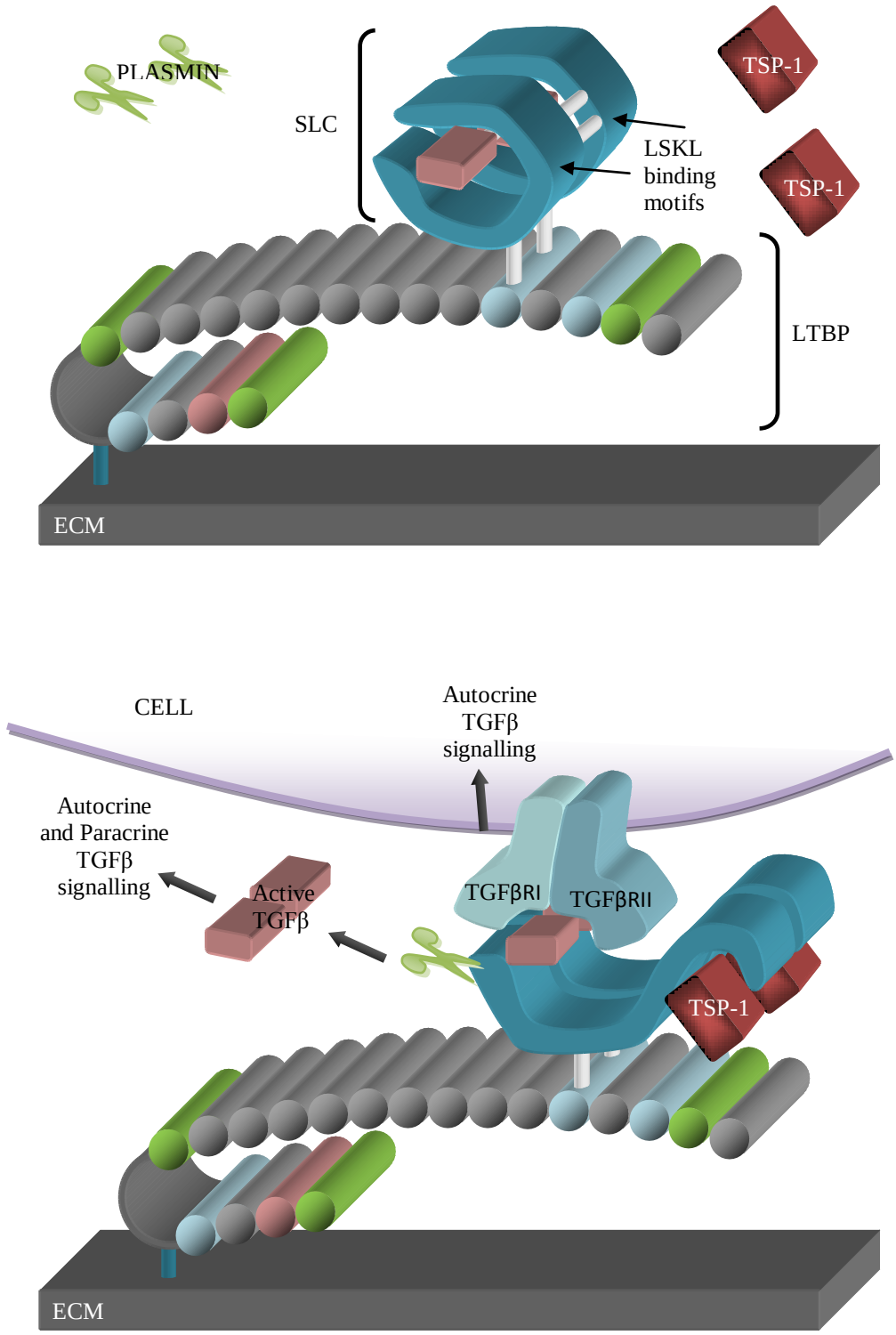

Figure 1.12. TSP-1 mediated activation of TGFβ. TSP-1 binds to LAP via an LSKL motif which causes a conformational change in LAP. This results in the exposure of active TGFβ, which can signal through TGFβ receptors in an autocrine fashion. Further processing of TGFβ by plasmin can cause the release of diffusible TGFβ. Diffusible TGFβ is able to induce both autocrine and paracrine TGFβ signalling. TSP-1 can also dock with its receptor CD36, which causes the complex to be localised to the cell surface. In the presence of CD36 the same processing and signalling responses can occur as described above. Adapted from information gleaned from the following sources (Schultz-Cherry and Murphy-Ullrich 1993; Crawford, Stellmach et al. 1998; Ribeiro, Poczatek et al. 1999; Yehualaeshet, O'Connor et al. 1999)

1.9.3. Disruption of TGFβ regulation

The LTBP targets the LLC to the ECM and consequently regulates the spatial availability of TGFβ. Mutation of LTBP causes a malfunction in the spatial regulation of TGFβ, where the lack of a particular LTBP isoform can result in inappropriate coupling of TGFβ with an alternate LTBP isoform resulting in modified TGFβ distribution in the ECM (Annes, Munger et al. 2003). Disruption of ECM components can also result in the misregulation of TGFβ activation. For example in Marfan syndrome (MFS) the gene encoding fibrillin-1 is mutated, LTBP is unable to target predefined areas of the ECM (i.e. fibrillin) and the LLC cannot be deposited in the matrix. This releases the LLC into the extracellular space where it is more vulnerable to processing. Spatially and temporally inappropriate activation of TGFβ ensues and as a consequence disproportionate bone growth and changes in vascularity occur (Ramirez and Dietz 2007; Ramirez and Rifkin 2009). Chaudhry and colleagues have shown that fibrillin fragments can inhibit the interaction of the LLC with the ECM and cause inappropriate activation of TGFβ (Chaudhry, Cain et al. 2007). This may constitute another level of control of TGFβ activation indicative of the homeostatic response involving a shift in complex deposition. TGFβ is therefore regulated on many levels, through intracellular post-translational modification, localisation to the ECM and through a variety of activation mechanisms.

1.9.4. TGFβ Signalling

Cell surface TGFβ receptors include type I and type II receptors (TGFβRI and TGFβRII) – these receptors have serine/threonine kinase activity. A third TGFβ receptor (TGFβRIII) can enhance binding of active TGFβ to TGFβRII. In the canonical signalling pathway, TGFβ binding to the type II receptor, type I and type II receptors co-localise to form heterotetrameric complexes. TGFβRII phosphorylates TGFβRI, leading to phosphorylation and subsequent activation of SMAD proteins (R-SMADs, 2 and 3) with the help of SMAD anchor for receptor activation (SARA), which co-ordinates R-SMAD docking at TGFβRI. Inhibitory SMAD (I-SMAD or SMAD-7) can negatively regulate the phosphorylation of R-SMADs. Once Phosphorylated R-SMADs dissociate from TGFβRI and SARA and bind to a co-SMAD (SMAD4), this complex translocates to the nucleus and effects transcriptional regulation of target genes with the association of co-activators. Co-repressors and co-stimulators inhibit and stimulate the regulation of target genes respectively and represent another level of transcriptional control. SMAD ubiquitination regulatory factors (SMURF) are important in the targeted ubiquitination of R-SMADs and work in association with I-SMAD to target receptors for ubiquitination, this prevents constitutive regulation of TGFβ target genes (for a more detailed review of TGFβ signalling see; (Shi and Massague 2003).

SMAD signalling is not the only signalling pathway stimulated downstream of TGFβ serine/threonine kinase receptors, a complex array of signalling pathways including mitogen activated protein kinases (MAPK) (p38, c-Jun N-terminal kinases [JNK] and extracellular signal related kinase [ERK]), phosphoinositol 3-kinase (PI3K) / Akt, nuclear factor κB (NFκB), Rho/ Rho associated protein kinase (ROCK), Ras and Notch are also stimulated by TGFβ (Moustakas and Heldin 2005). Since TGFβ is known to play a major role in wound healing and fibrosis, knowledge of its role and regulation in tendon is likely to be important in understanding the cause and progression of tendinopathy.

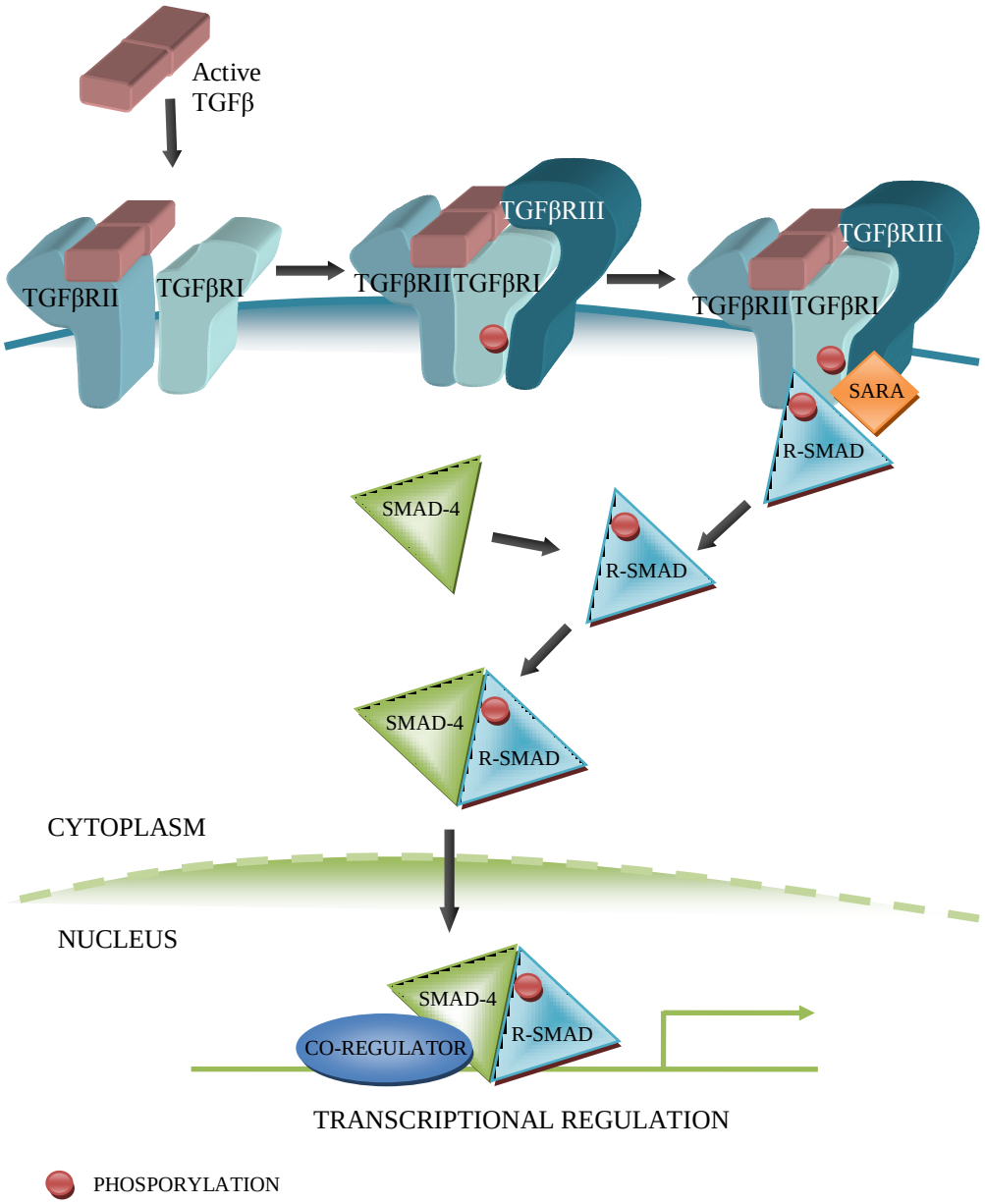

Figure 1.13. TGFβ Signalling: An overview. Upon TGFβ ligand binding to the type II receptor, type I and type II receptors co-localise to form a heterotetrameric complex (TGFβRI and RII are shown here in a single entity instead of duplicate). TGFβRIII functions to stabilise this interaction. TGFβRII phosphorylates TGFβRI, leading to phosphorylation and subsequent activation of SMAD proteins (R-SMADs, 2 and 3) with the help of SMAD anchor for receptor activation (SARA), which co-ordinates R-SMAD docking at TGFβRI. Once Phosphorylated R-SMADs dissociate from TGFβRI and SARA and bind to a co-SMAD (SMAD4), this complex translocates to the nucleus and effects transcriptional regulation of target genes with the association of co-activators and co-repressors. Adapted from (Shi and Massague

1.9.5. TGFβ and mechanical load

TGFβ expression as well as TGFβ receptor synthesis and regulation of activity is sensitive to mechanical loading in a range of cell types; Latent and active TGFβ1 and latent TGFβ3 were increased following cyclic stretching in rat mesangial cells (Riser, Cortes et al. 1996); mechanical stimulation induced the synthesis and expression of TGFβ receptor I and II in a similar model (Riser, Ladson-Wofford et al. 1999). In cell studies of endothelium and anterior cruciate ligament mechanical load (fluid shear and tensional forces models respectively) induced at least a 50% increase in the level of TGFβ protein expression (Kim, Akaike et al. 2002; Baker, Ettenson et al. 2008).

In human patellar tendon there have been reports of increased levels of TGFβ mRNA and protein in response to mechanical loading in monolayer cell culture systems. Cyclic strain regimes ranging from 4-8% at 0.5-1Hz showed a 25-64% increase in TGFβ at the protein level and 11.5-24.6% increase at the mRNA level, with more robust changes seen with higher loading regimes (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004). However these studies did not confirm the activity of TGFβ or which isoforms were regulated. In a murine Achilles tendons cell culture model, TGFβ activity was increased in cells exposed to fluid shear stress, however the specific isoform involved was not measured (Maeda, Sakabe et al. 2011). In addition a study by Legerlotz and colleagues showed no change in the level of TGFβ mRNA in response to mechanical load in an in vivo model of rat strength training (Legerlotz, Schjerling et al. 2007). However as the levels total and active protein were not measured we cannot rule out effects upon TGFβ signalling. Therefore TGFβ is increased in response to strain in a range of tissues and different type of strain at the mRNA, protein and activity levels. However, stress deprivation of tendon can also induce an increase in TGFβ at the protein level (Uchida, Tohyama et al. 2005), whether this is active and capable of signalling remains to be elucidated.

Kjaer et al (Kjaer, Langberg et al. 2009) proposed TGFβ may also play a role in the modulation of the adaptive response to strain, as TGFβ regulates collagen (Heinemeier, Olesen et al. 2009). Therefore TGFβ may play a role in the regulation of major components of the ECM, which may lead to stabilisation of the matrix in response to load.

1.9.6. TGFβ and tendinopathy

An increase in Transforming Growth Factor β (TGFβ) has been reported in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002); although the activity of these proteins was not confirmed, and in the absence of TGFβRI the TGFβ signalling cascade was not activated (Fenwick, Curry et al. 2001). This highlights TGFβ signalling as a possible candidate in the detection of mechanical stimulation and disruption of this intimate relationship could result in tendinopathy. Therefore investigating the role of TGFβ in the tendon response to mechanical loading could prove invaluable in the understanding of tendinopathy

Aims:

We hypothesise that moderate / high mechanical loading will induce anabolic changes in metalloproteinase and matrix genes and that the regulation of these genes may involve TGFβ. We aim to measure gene expression of a broad range of metalloproteinase and matrix genes to test whether they are regulated in an anabolic manner. Secondly, to further characterise mechanotransduction by the addition of inhibitors of an array of signalling pathways; assessment of gene regulation in response to strain with the addition of these inhibitors will confirm whether the signalling pathways targeted by these inhibitors are involved in mechanoregulation of gene expression.

CHAPTER 2; Materials and Methods

2.1. MATERIALS

Acetone	Fisher, Leicestershire
Agar	Fisher, Leicestershire
Agarose	Sigma Aldrich, Dorset
All Stars negative control siRNA	Qiagen, Crawley
Amiloride hydrochloride	Sigma Aldrich, Dorset
Ampicillin	Sigma Aldrich, Dorset
Analytical Grade Water	Fisher, Leicestershire
Apyrase	Sigma Aldrich, Dorset
Avidin-HRP	BioRad, Hertfordshire
Biotin rat anti-mouse, human, pig anti-TGF β1 (555053)	BD Biosciences, Oxford
Bovine Serum Albumin	Sigma Aldrich, Dorset
Brij 35	Fisher, Leicestershire
Bromophenol blue	Sigma Aldrich, Dorset
Calcium Chloride	Fisher, Leicestershire
Calcein AM	Fisher, Leicestershire
CDP star reagent	Sigma Aldrich, Dorset
Chloroform	Fisher, Leicestershire
Cryo-M-Bed embedding compound	Bright, Huntington
Coomassie Brilliant Blue	Sigma Aldrich, Dorset
Dharmafect 1	Fisher, Leicestershire
DMEM	Fisher, Leicestershire
dNTP mix	Promega, Southampton

Doramapimod	Cambridge Bioscience
Dual luciferase reporter assay kit	Promega, Southampton
Ethanol	Fisher, Leicestershire
Ethidium Homodimer	Fisher, Leicestershire
Fibrinogen from bovine plasma	Sigma Aldrich, Dorset
GAP27	Severn Biotech Ltd.
Glycerol	Fisher, Leicestershire
Glycine	Fisher, Leicestershire
Glycogen, Molecular biology grade	Sigma Aldrich, Dorset
GM6001	Millipore, Watford
GM6001 inactive control	Merck Chemicals Ltd.
GRGDSP (RDG) peptide inhibitor	Cambridge Bioscience
GRGESP control peptide	Cambridge Bioscience
Hirudin	Sigma Aldrich, Dorset
Human hsa-miR24 (primer set)	Life technologies, Paisley
Human IL6 siRNA	Qiagen, Crawley
Human SMAD4 siRNA	Qiagen, Crawley
Human TGFβ1 siRNA	Qiagen, Crawley
Human TGFβ2 siRNA	Qiagen, Crawley
Human TGFβ3 siRNA	Qiagen, Crawley
Hydrochloric Acid	Fisher, Leicestershire
IL6 antibody	R&D systems, Abingdon
Indomethacin	Sigma Aldrich, Dorset
Isopropanol	Fisher, Leicestershire
KAPA Probe fast qPCR kit Mastermix	Anachem, Bedfordshire
LEAF^{TM} purified rat IgG2a κ isotype control antibody	Cambridge Bioscience
Lipofectamine 2000	Life technologies, Paisley
LSKL mimetic peptide	Cambridge Bioscience
MAB13 antibody against integrin β1 (Prof Martin Humphries, Manchester)	

Mannose-6-Phosphate	Sigma Aldrich, Dorset
Methanol	Fisher, Leicestershire
Milk powder	Fisher, Leicestershire
Na HPO_4 (Sodium phosphate)	Fisher, Leicestershire
NaN_2P0_4	Fisher, Leicestershire
Nifedipine	Sigma Aldrich, Dorset
On target plus smartpool human ADAM12 siRNA	Abgene Ltd.
PD0325901	Cambridge Bioscience
Pefabloc	Sigma Aldrich, Dorset
Penicillin / Streptomycin	Fisher, Leicestershire
Pepstatin	Roche, West Sussex
Phenol free DMEM	Fisher, Leicestershire
PI-103	Cambridge Bioscience
Protease inhibitor cocktail	Sigma Aldrich, Dorset
Purified rat anti-mouse, human, pig TGFβ1(Capture, 555052)	BD Biosciences,Oxford
PVDF	Millipore, Watford
QF24 substrate	Enzo life sciences, Exeter
Random Hexamers	Life technologies, Paisley
Rat tail type I collagen (2.2mg/ml)	First Link, Birmingham
Recombinant IL6	R&D systems, Abingdon
Recombinant TGFβ	R&D systems, Abingdon
Recombinant MMP2	R&D systems, Abingdon
RNAsin Ribonuclease inhibitor	Promega, Southhampton
SB203580	Cambridge Bioscience
SLLK peptide (unspecific control)	Cambridge Bioscience
Sodium Chloride	Fisher, Leicestershire
Sodium dodecyl sulfate	Fisher, Leicestershire
Sodium Hydroxide	Sigma Aldrich, Dorset
Sulphuric acid	Fisher, Leicestershire

Superscript II Reverse Transcription kit	Life technologies, Paisley
Tetramethylbenzidine (TMB)	Sigma Aldrich, Dorset
TGFβRI inhibitor - SB431542 hydrate	Sigma Aldrich, Dorset
TGFβ1 antibody (MAB240)	R&D systems, Abingdon
TGFβ2 antibody (AB-122-NA)	R&D systems, Abingdon
TGFβ3 antibody (MAB243)	R&D systems, Abingdon
Thapsigargin	Sigma Aldrich, Dorset
Thrombin	Sigma Aldrich, Dorset
Tris	Fisher, Leicestershire
Trizol	Sigma Aldrich, Dorset
Trypsin / EDTA (0.25%)	Fisher, Leicestershire
Tryptone	Fisher, Leicestershire
Tween 20	Sigma Aldrich, Dorset
U-0126	Cambridge Bioscience
U73122 hydrate	Sigma Aldrich, Dorset
Universal PCR mastermix, No Amperase UNG	Life technologies, Paisley
Wortmannin	Cambridge Bioscience
Yeast extract	Fisher, Leicestershire
α18-glycyrrhetinic acid	Sigma Aldrich, Dorset
β-mercaptoethanol	Sigma Aldrich, Dorset
TESPA (3'aminopropyl-triethoxy silane)	Sigma Aldrich, Dorset
Triton-X100	Sigma Aldrich, Dorset
Bovine Serum Albumin	Sigma Aldrich, Dorset
Paraformaldehyde	Fisher, Leicestershire
Vectashield	Fisher, Leicestershire

2.2 CELL CULTURE METHODS

2.2.1 Cell culture

Human Achilles tenocytes derived from patients with Achilles tendinopathy were grown by explant outgrowth from small pieces of tendon cultured in Dulbecco's modified Eagle's medium (DMEM) (with sodium pyruvate, phenol red, glutamax™ and low glucose,) containing heat inactivated foetal bovine serum and penicillin (10mg/ml) / streptomycin (10u/ml) at a ratio of 100:10:1. Medium was replaced every 3 to 4 days until cells reached confluence. Cells were passaged using Trypsin / EDTA (0.25%) and divided into cell culture flasks at an average ratio of 1:4. Cells were cultured for up to 9 passages, cells later than passage 10 were deemed to have differentiated from the tenocyte phenotype.

Cells were cryogenically frozen by; trypsinisation, centrifugation at 150 x g for 5 minutes and re-suspension in foetal bovine serum (+10% DMSO) before incubation overnight in a cell freezing chamber at -80°C. Cells were then transferred to liquid nitrogen for longer term storage.

2.2.2 Tissue Train cell culture protocol

Tenocytes were grown to ~90% confluence in order to avoid contact inhibition during the experimental setup. Cells were trypsinised and re-suspended at either $1x10^6$ or $3x10^6$ cells/ml (double the final density) in serum free medium (1% penicillin [10mg/ml] / streptomycin [10u/ml]). Rat tail type I collagen (2.2mg/ml) was mixed with 10x DMEM at a ratio of 9:1 and the pH was adjusted to approximately 7 by the addition of 10M Sodium Hydroxide (NaOH), using Phenol red indicator (Orange/red colour indicates a pH of approximately 7) to assesss the pH. Neutralised collagen and tenocyte suspension were mixed 1:1 (1mg/ml collagen, $0.5x10^6$ and $1.5x10^6$ cells/ml for cell density experiments and $1.5x10^6$ cells/ml thereafter, final density) and 200µl was pippetted into flexible bottomed collagen type I coated Flexcell™ tissue train plates (Dunn Labortechnik, Asbach, Germany). During which time the plates were under a continuous vacuum (20% elongation of the rubber membrane) over a trough loader (circular posts with a grove to allow the gel to rest during polymerisation, see Fig. 2.1) and base plate. The vacuum was controlled by the Flexcell® FX-4000™ Tension System consisting of a computer running FX-4000™ software, a vacuum controller and a vacuum pump. The computer software regulates the vacuum controller which regulates the vacuum pump to pull a vacuum from the base plate. Gels (plus tenocytes) were allowed to set for 1 hour at 37 ^oC under a constant vacuum

(20% elongation), at which point 3ml serum-free DMEM was added (1% penicillin [10mg/ml] / streptomycin [10u/ml]). After a further 72 hours medium was replaced and appropriate treatments added (See individual chapters and materials list for details). Tissue train plates were transferred onto arctangle loading posts (rectangle posts with arc shaped edges to fit into the circular well, see Fig. 2.1) in preparation for mechanical loading (Garvin, Qi et al. 2003). A uniaxal strain was applied using the Flexcell FX-4000^{TM} tension system (non-strain controls included). The uniaxial strain applied was in sinusoidal wave form cycling between 0 and 5% elongation at 1 Hz for up to 48 hours. Uniaxial strain in sinusoidal wave form was chosen due to the similarity to the physiological movement of tendon, i.e. the smooth uniaxial movement during steady excercise such as walking. The frequency of 1 Hz was chosen to simulate walking pace.

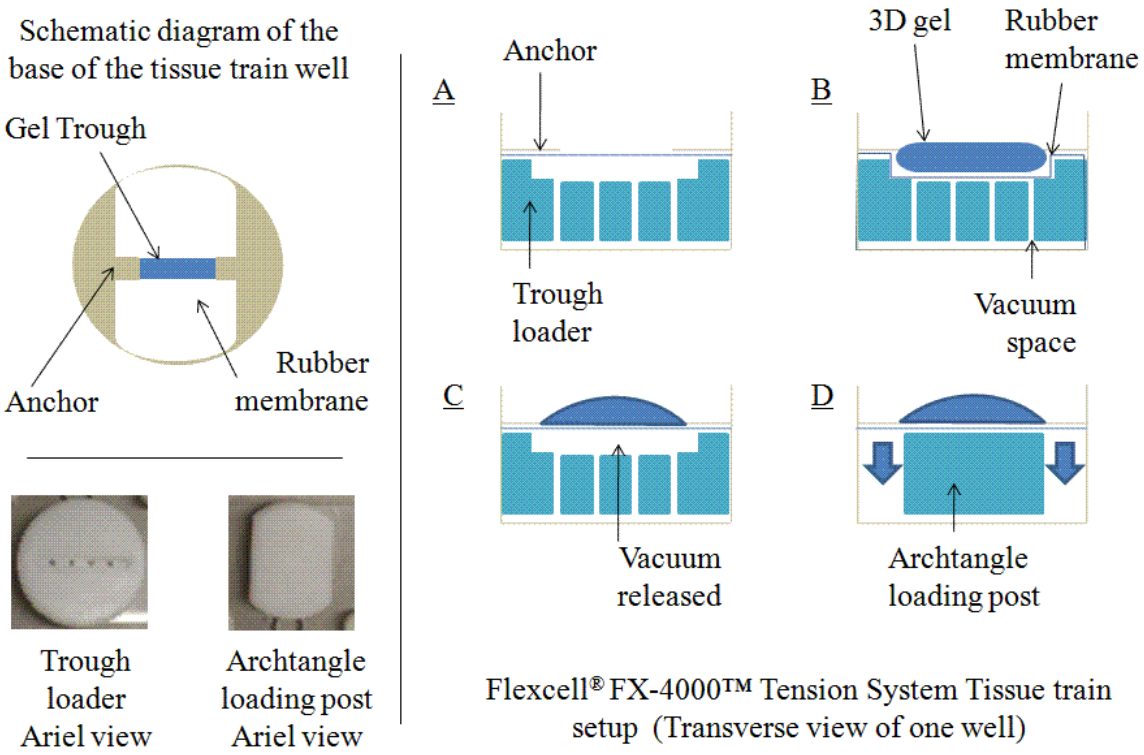

Figure 2.1. Flexcell FX-4000™ tension system tissue train setup. Flexcell™ tissue train plates are 6 well plates each with a flexible rubber membrane at its base. At either side of each well are fibrous anchors (A). When the tissue train plate is placed upon the trough loader and a vacuum is applied (20% elongation) the rubber membrane is pulled into the gel trough, the collagen gel (plus tenocytes) can then be seeded into the trough (B). 1 hour incubation at 37°C allows the gel to polymerise. The vacuum is released (C) and the tissue train plate is transferred to the arctangle loading post, the collagen gel (plus tenocytes) remains attached to either side of the well via fibrous anchors. Applying a vacuum causes the rubber membrane to deform and stretches the gel uniaxially between the fibrous attachments (D). Adapted from (Garvin, Qi et al. 2003).

2.2.3 Cell viability testing – Calcein AM and Ethidium Homodimer staining

To determine the viability of tenocytes after mechanical loading, 3D collagen gels seeded with primary Achilles human tenocytes strained for 48 hours using the Flexcell^{TM} tissue train cell culture system; 3D collagen gels were stained using Calcein AM, which localises to the cytoplasm of viable cells and Ethidium homodimer, which binds to DNA in the nucleus of dead cells. In brief; 3D collagen gels were washed in PBS and incubated in phenol red - free DMEM containing both Calcein AM and ethidium homodimer (50µg/ml and 50µM respectively)) for 45 minutes at 37°C. Gels were transferred to slides and coverslips were mounted for viewing on the charge-coupled device (CCD) upright Zeiss fluorescence microscope with an attached digital camera and Axiovision 4.7 software (Zeiss, Oberkochen, Germany). Calcein AM emits green fluorescence (500-530 nm) when excited with blue light (488 nm) and ethidium homodimer emits a red fluorescence (600-650 nm) when excited with green light (564nm).

2.2.4 Cell viability testing – tenocyte extraction by collagenase digestion

To determine the viability of tenocytes after mechanical loading 3D collagen gels seeded with primary human Achilles tenocytes strained for 48 hours using the Flexcell^{TM} tissue train system; 3D gels were washed in PBS and digested in collagenase from Clostridium Histolyticum (10mg/ml) for 5 hours (0.1% Collagenase, 0.4% HEPES in DMEM). Cells were centrifuged at 150 x g for 5 minutes, supernatant was removed and cells were re-suspended in 200µl of medium. Cells were stained with trypan blue (cells stained blue are non-viable) and counted using a haemocytometer. Numbers of live and dead cells were quantitated, comparisons were made between cells treated ±strain.

2.2.5 siRNA knockdown

Primary Achilles tenocytes were grown to confluence in DMEM containing heat inactivated foetal bovine serum (10%) and penicillin / streptomycin 1%. Medium was removed and cells were washed in PBS to remove all traces of penicillin / streptomycin. Medium was replaced with DMEM containing 10% heat inactivated foetal bovine serum without penicillin / streptomycin.

siRNA (Human IL6, SMAD4, TGFβ1, TGFβ2, TGFβ3 siRNA [Qiagen, Crawley]) and Dharmafect 1 (transfection reagent) were equilibrated at room temperature for 5 minutes, mixed together and incubated for a further 20 minutes. The resulting transfection medium

was incubated with cells for 48 hours before cells were trypsinised and utilised in the tissue train cell culture protocol. The final concentrations of siRNA and transfection reagent were 50nM and 0.2% respectively. And all DMEM used during this protocol contained 10% heat inactivated foetal bovine serum in the absence of penicillin / streptomycin.

2.3 MOLECULAR ANALYSIS METHODS

2.3.1 RNA extraction and Reverse Transcription

Tenocyte-seeded collagen gels were dissolved in Trizol reagent (4x gel volume). RNA was isolated as described previously using a tri-spin protocol (Ireland, Harrall et al. 2001). In brief, 125ug/ml glycogen was added and incubated at room temperature for 3 minutes (glycogen binds to RNA and allows the observation of a clear pellet at the latter stages of this protocol). 2/5 sample volume of chloroform was added to the samples which were shaken vigorously for 15 seconds then incubated at room temperature for 5 minutes. After centrifugation (at 12000 x g for 15 minutes) the upper phase was transferred into a fresh 1.5ml tube, an equal volume of isopropanol was added, and samples were incubated at room temperature for at least 10 minutes. Samples were centrifuged for 10 minute at 12000 x g and the supernatant was discarded. Pellets were washed with 1ml ethanol (vortexed then centrifuged for 5 minutes at 7500 x g before the ethanol was removed), air dried and re-suspended in 50ul of analytical grade water. The RNA concentration of samples was estimated using a nanodrop spectrophotometer. The absorbance ratio A_{260}:A_{280} was 1.76 ± 0.005 (mean ± S.E.M.) with an average concentration of 43ng/µl ± 0.5 (mean ± S.E.M.). This confirms that RNA was of good quality. RNA was diluted to 23-40ng/µl depending on the experimental set in preparation for reverse transcription.

RNA was primed with random hexamers and reverse transcribed with the superscript II kit according to manufacturer's instructions. In brief, RNA was incubated at 70^{o}C for 10 minutes with 200ng random hexamers. 4µl 5x sample buffer, 10mM DTT, 0.125mM dNTP's (2.5mM), 200units Superscript II and 40units RNase inhibitor were added and incubated for 1 hour at 42^{o}C and at 70 ^{o}C for 10 minutes (all reagents apart from the random hexamers are from the superscript II kit).

2.3.2 Primer Design

Primers were designed using Primer Express software (Applied Biosystems). Primer specificity and dimerisation were estimated using the Primer-BLAST tool (NCBI website - http://www.ncbi.nlm.nih.gov). Newly synthesised primers were used to amplify the gene of interest using SYBR green RT-PCR and melt curve analysis was performed to determine unspecific amplification or primer dimerisation. Agarose gel electrophoresis and

oligonucleotide sequencing were used to confirm the correct amplicon size and sequence respectively.

2.3.3 Quantitative Real Time PCR

The standard qRT-PCR programme was run using selected primer probe sets (see Table 2.1) and the Applied biosystems 7500 real time PCR system. Each reaction was performed in a volume of 25µl including; 11-19ng/well of cDNA (depending on the experimental setup), 33% KAPA Probe fast qPCR kit Mastermix (2x), 0.2 nM each of the forward and reverse primer and 0.1 n*M* of probe. The thermal cycles were as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Pooled cDNA products from each experiment were serially diluted to create a standard curve. Standard curves were run for each assay to confirm primer probe efficiency. As the standard curves were 1:5 serial dilutions the expected slope was -3.8 and the Correlation coefficient or R^2 value expected was 1, only standard curves with similar values to this were accepted (slope ±0.5, R^2 ±0.1). Relative expression levels in each gene of interest were analysed by normalising to endogenous control genes *Topoisomerase-1 (TOP1*) or *18s* (ΔCt [endogenous control gene Ct-gene of interest Ct]) and linearising these data by expressing it as $2^{\Delta Ct}$. Alternatively data was expressed as a fold change from the control ($2^{\Delta\Delta Ct}$ [treatment condition $2^{\Delta Ct}$ / Control $2^{\Delta Ct}$). Data shown was normalised to *TOP1,* as GeNorm analysis demonstrated *TOP1* to be the most stable housekeeping gene (normalising to 18s yielded similar results).

Gene	Sequences	Reference
MMP1	Forward Primer: 5'- AAGATGAAAGGTGGACCAACAATT -3' Reverse Primer: 5' -CCAAGAGAATGGCCGAGTTC -3' Probe: 5'- FAM-CAGAGAGTACAACTTACATCGTGTTGCGGCTC-TAMRA -3'	(Clark, Young et al. 2010)
MMP3	Forward Primer: 5'- TTCCGCCTGTCTCAAGATGATAT -3' Reverse Primer: 5'- AAAGGACAAAGCAGGATCACAGTT -3' Probe: 5'- FAM-TCAGTCCCTCTATGGACCTCCCCCTGAC-TAMRA -3'	(Nuttall, Pennington et al. 2003)
MMP13	Forward Primer: 5'- AAATTATGGAGGAGATGCCCATT -3' Reverse Primer: 5'- TCCTTGGAGTGGTCAAGACCTAA -3' Probe: 5'- FAM-CTACAACTTGTTTCTTGTTGCTGCGCATGA-TAMRA -3'	(Nuttall, Pennington et al. 2003)
ADAM12	Forward Primer: 5'- AGCTATGTCTTAGAACCAATGAAAAGTG –3' Reverse Primer: 5'- CCCCGGACGCTTTTCAG –3' Probe: 5'- FAM-ACCAACAGATACAAACTCTTCCCAGCGAAGA-TAMRA –3'	(Jones, Corps et al. 2006)
COL1A1	Forward primer: 5' –CTGGTCACCATGGTGATCAAG– 3' Reverse primer: 5' –GCAGGCGGGAGGACTTG– 3' Probe: 5' – CTGTCGATGGCTGCACGAGTCACAC – TAMRA – 3'	(Ireland, Harrall et al. 2001)
ADAMTS5	Forward primer: 5'-TGTCCTGCCAGCGGATGT-3' Reverse primer: 5'-ACGGAATTACTGTACGGCCTACA-3' Probe: 5'- FAM-TTCTCCAAAGGTGACCGATGGCACTG-TAMRA -3'	(Porter, Scott et al. 2004)
TGFβ1	Forward primer: 5'-TGAGGGCTTTCGCCTTAGC-3' Reverse primer: 5'-CGGTAGTGAACCCGTTGATGT-3' Probe: 5'- FAM-CTCCTGTGACAGCAGGGATAACACACTGC-TAMRA-3'	Own Design
TGFβ2	Forward primer: 5'-ACGGATTGAGCTATATCAGATTCTCA-3' Reverse primer: 5'-AACAGCATCAGTTACATCGAAGGA-3' Probe: 5'- FAM-TTTAACATCTCCAACCCAGCGCTACATCG-TAMRA-3'	Own Design
TGFβ3	Forward primer: 5'-TGTCACACCTTTCAGCCCAAT-3' Reverse primer: 5'-CTCCACGGCCATGGTCAT-3' Probe: 5'- FAM-ATTGTCCACGCCTTTGAATTTGATTTCCAT-TAMRA-3'	Own Design
TOP1	Perfect probe reference gene assay from Primer design	Primer design kit
18s	Forward primer: 5'-GCCGCTAGAGGTGAAATTCTTG-3' Reverse primer: 5'-CATTCTTGGCAAATGCTTTCG-3' Probe: 5'-ACCGGCGCAAGACGGACCAG-3'	(Corps, Robinson et al. 2006)

Table 2.1. Quantitative Real Time PCR primer probe sets. Primer and probe sets are listed for the genes analysed using qRT-PCR. Our own design primer probe sets were designed using the Primer Express software (Applied Biosystems). The fluorophore used was FAM and the quencher used was TAMRA.

2.3.4 Taqman Low Density Array (TLDA) analysis

The TLDA (Applied Biosystems) was designed to assess all 23 MMP genes as well as 18 of the 19 currently identified ADAMTS genes (our study excludes ADAMTS20 due to the lack of space on the TLDA plate), all 4 TIMP genes, 10 selected Proteoglycan (known to be expressed in tendon) and 4 collagen genes (known to be expressed in tendon), as well as the endogenous control gene 18s (See Table 2.2 & Table 2.3). 50ng (Metalloproteinase and TIMP family analysis TLDA) or 243ng (Time course of selected genes TLDA) (concentration dependant on available cDNA) cDNA and universal PCR mastermix (50µl) were loaded into the fill reservoirs (100µl/reservoir) and the plate was run according to manufacturer's instructions, using the Applied biosystems 7900HT Real-Time PCR System and Applied biosystems Sequence Detection Systems (SDS 2.3 and RQ manager 1.2) software. The thermal cycles were as follows: 50°C for 2 minutes, 94.5°C for 10 minutes followed by 40 cycles of 97°C for 30 seconds and 59.7°C for 1 minute. Details of the primer, probe sets chosen are listed in Tables 2 (metalloproteinase and TIMP family analysis TLDA) and 3 (Time course of selected genes TLDA). Undetected samples were given an arbitrary Ct value of 40. Where the average Ct for a particular gene was ≥37 the level was deemed too low for detection Relative expression levels in each gene of interest were analysed by normalizing to endogenous control gene *18s* (ΔCt [endogenous control gene Ct-gene of interest Ct]) and expressing these data as $2^{\Delta\Delta Ct}$ (fold change with strain and / or TGFβ).

Metalloproteinase and TIMP TLDA		
Primer probe set	Gene Abbreviation	Full name
Hs99999901_s1	18S	18s rRNA
Hs00909449_m1	ACTA2	α Smooth Muscle Actin
Hs99999905_m1	GAPDH	**Glyceraldehyde 3-phosphate dehydrogenase**
Hs00199608_m1	ADAMTS1	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 1
Hs00247973_m1	ADAMTS2	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 2
Hs00610744_m1	ADAMTS3	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 3
Hs00192708_m1	ADAMTS4	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 4
Hs00199841_m1	ADAMTS5	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 5
Hs01058097_m1	ADAMTS6	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 6
Hs00276223_m1	ADAMTS7	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 7
Hs00199836_m1	ADAMTS8	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 8
Hs00172025_m1	ADAMTS9	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 9
Hs00372835_m1	ADAMTS10	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 10
Hs00229594_m1	ADAMTS12	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 12
Hs00260148_m1	ADAMTS13	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 13
Hs00365506_m1	ADAMTS14	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 14
Hs00373520_m1	ADAMTS15	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 15
Hs00373526_m1	ADAMTS16	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 16
Hs00330236_m1	ADAMTS17	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 17
Hs00373501_m1	ADAMTS18	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 18
Hs00999225_m1	ADAMTS19	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 19
Hs00899658_m1	MMP1	Matrix MetalloProteinase 1
Hs00234422_m1	MMP2	Matrix MetalloProteinase 2
Hs00968308_m1	MMP3	Matrix MetalloProteinase 3
Hs01042795_m1	MMP7	Matrix MetalloProteinase 7
Hs01029057_m1	MMP8	Matrix MetalloProteinase 8
Hs00957562_m1	MMP9	Matrix MetalloProteinase 9
Hs00233987_m1	MMP10	Matrix MetalloProteinase 10
Hs00171829_m1	MMP11	Matrix MetalloProteinase 11
Hs00899662_m1	MMP12	Matrix MetalloProteinase 12
Hs00233992_m1	MMP13	Matrix MetalloProteinase 13
Hs00237119_m1	MMP14	Matrix MetalloProteinase 14
Hs00233997_m1	MMP15	Matrix MetalloProteinase 15
Hs01095537_m1	MMP16	Matrix MetalloProteinase 16
Hs00211754_m1	MMP17	Matrix MetalloProteinase 17
Hs00275699_m1	MMP19	Matrix MetalloProteinase 19
Hs01573770_m1	MMP20	Matrix MetalloProteinase 20
Hs00377680_m1	MMP21	Matrix MetalloProteinase 21
Hs00270380_m1	MMP23B;MMP23A	Matrix MetalloProteinase 23
Hs00198580_m1	MMP24	Matrix MetalloProteinase 24
Hs01554789_m1	MMP25	Matrix MetalloProteinase 25
Hs00983740_m1	MMP26	Matrix MetalloProteinase 26
Hs00223193_m1	MMP27	Matrix MetalloProteinase 27
Hs01020031_m1	MMP28	Matrix MetalloProteinase 28
Hs00171558_m1	TIMP1	Tissue Inhibitor of MetalloProteinase 1
Hs00234278_m1	TIMP2	Tissue Inhibitor of MetalloProteinase 2
Hs00165949_m1	TIMP3	Tissue Inhibitor of MetalloProteinase 3
Hs00162784_m1	TIMP4	Tissue Inhibitor of MetalloProteinase 4

Table 2.2. Taqman Low Density Array primer sets: Metalloproteinase and TIMP family analysis TLDA. Primer and probe sets are listed for the genes analysed using qRT-PCR.

Time course TLDA		
Primer probe set	Gene Abbreviation	Full name
Hs99999901_s1	18S	18s rRNA
Hs00243257_m1	TOP1	Topoisomerase 1
Hs00153936_m1	ACAN	**Aggrecan**
Hs00559403_m1	ACTA1	α Smooth Muscle Actin
Hs00192708_m1	ADAMTS4	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 4
Hs00199841_m1	ADAMTS5	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 5
Hs01058097_m1	ADAMTS6	A Disintegrin And Metalloproteinase with Thrombospondin Motifs 6
Hs00372835_m1	ADAMTS10	A Disintegrin And Metalloproteinase with Thrombospondin Motifs10
Hs00373526_m1	ADAMTS16	A Disintegrin And Metalloproteinase with Thrombospondin Motifs16
Hs00959143_m1	BGN	Biglycan
Hs00164004_m1	COL1A1	Collagen, type I, alpha 1
Hs00156568_m1	COL2A1	Collagen, type II, alpha 1
Hs00943809_m1	COL3A1	Collagen, type III, alpha 1
Hs00189184_m1	COL12A1	Collagen, type XII, alpha 1
Hs00966234_m1	COL14A1	Collagen, type XIV, alpha 1
Hs00164359_m1	COMP	Cartilage Oligomeric matrix protein
Hs00170014_m1	CTGF	Connective Tissue Growth Factor
Hs00370384_m1	DCN	Decorin
Hs00171191_m1	FBN1	Fibronectin
Hs00277509_m1	FN1	Fibrillin-1
Hs00168275_m1	GRM5	Metabotropic glutamate receptor 5
Hs00968692_m1	GRM6	Glutamate receptor, metabotropic 6
Hs00157103_m1	HAPLN1	Link Protein
Hs01547656_m1	IGF1	Insulin-like growth factor 2
Hs00174383_m1	IL17A	Interleukin 17A
Hs00174097_m1	IL1B	Iinterleukin 1B
Hs00174122_m1	IL4	Interleukin 4
Hs00985639_m1	IL6	Interleukin 6
Hs01075667_m1	IL6R	Interleukin 6 Receptor
Hs00158940_m1	LUM	Lumican
Hs00899658_m1	MMP1	Matrix MetalloProteinase 1
Hs00234422_m1	MMP2	Matrix MetalloProteinase 2
Hs00968308_m1	MMP3	Matrix MetalloProteinase 3
Hs01029057_m1	MMP8	Matrix MetalloProteinase 8
Hs00233987_m1	MMP10	Matrix MetalloProteinase 10
Hs00233992_m1	MMP13	Matrix MetalloProteinase 13
Hs00153133_m1	PTGS2	Cyclooxygenase 2
Hs03054634_g1	SCXB;SCXA	Scleraxis
Hs00165814_m1	SOX9	SOX9
Hs00998133_m1	TGFB1	Transforming Growth Factor β1
Hs00170236_m1	THBS1	Thrombospondin-1
Hs99999139_m1	TIMP1	Tissue Inhibitor of MetalloProteinase 1
Hs00234278_m1	TIMP2	Tissue Inhibitor of MetalloProteinase 2
Hs00165949_m1	TIMP3	Tissue Inhibitor of MetalloProteinase 3
Hs01115665_m1	TNC	Tenascin C
Hs99999043_m1	TNF	Tumour Necrosis Factor
Hs00223332_m1	TNMD	Tenomodulin
Hs01007933_m1	VCAN	Versican

Table 2.3. Taqman Low Density Array primer sets: Time course analysis of selected genes TLDA. Primer and probe sets are listed for the genes analysed using qRT-PCR.

2.4 PROTEIN ANALYSIS METHODS

2.4.1 TGFβ activity analysis

TGFβ can be detected using a cell based luciferase assay. Activation of SMAD proteins is a key step in the TGFβ signalling cascade. Cells transfected with a plasmid containing a construct that specifically binds to phosphorylated SMAD proteins and a luciferase reporter was used to detect TGFβ signalling. The construct binds to activated SMAD proteins, which induces the production of luciferase. Cell lysis releases the luciferase from confinement within the cell. In order to measure the level of luciferase produced, Luciferin was added. Luciferase cleaves Luciferin and a proportional increase in fluorescence was measured. In this way conditioned medium was assessed for active TGFβ. Medium was heat activated before incubation with the transfected cells to calculate total TGFβ. A transfection control plasmid was also used (Renilla), which produced luciferase upon entering the cell. The TGFβ luciferase assay was conducted essentially as described by (Jonk, Itoh et al. 1998). However the protocol used is described in detail below. Confirmation of the specificity of this assay is shown in section 3.3 of the optimisation chapter. In brief, we have shown that the Pan TGFβ antibody and the TGFβRI inhibitor abrogate the detection of the SMAD activation in strain or non-strain conditioned media from multiple experiments. This indicates that the assay is specifically detecting TGFβ and not BMPs.

Preparation of competent DH5α cells

E. Coli DH5α cells were spread on agar plates supplemented with 100µg/µl ampicillin and incubated overnight at 37°C. A single colony was inoculated into 5ml of LB broth (1% w/v Tryptone, 2% w/v Yeast Extract and 0.5% w/v Sodium Chloride) and incubated overnight at 37°C whilst shaking. Cells were grown to optimum density (OD_{600} = 0.6) and chilled on ice for 10 minutes (25ml tubes). Cells were centrifuged at 1000 x g for 10 minutes at 4°C and supernatant was removed. The cell pellet was re-suspended in 5ml 0.1M CaCl and incubated on ice for 10 minutes. The suspension was centrifuged at 1000 x g for 10 minutes at 4°C and supernatant was removed. The pellet was re-suspended in 1ml ice cold 0.1M CaCl and glycerol (400µl / ml) and stored frozen at -80°C.

Transformation of DH5α competent cells

CAGA or Renilla plasmid was added to 50µl suspension of competent DH5α cells at a ratio of 1:50; cells were incubated for 15 minutes on ice, 42°C for 32 seconds and 2 minutes on ice. DH5α cells were spread onto agar plates (1% w/v Tryptone, 2% w/v Yeast Extract, 0.5% w/v Sodium Chloride and 1.6% w/v Agar) and incubated overnight at 37°C. Individual colonies were picked and transferred to 5ml vials of LB broth (1% w/v Tryptone, 2% w/v Yeast Extract and 0.5% w/v Sodium Chloride) along with Ampicillin 100µg/ml and incubated overnight on a shaking device at 37°C.

Plasmid extraction (Qiagen mini preps)

Plasmids were extracted using a Qiagen mini prep kit following manufacturer's instructions. In brief, bacterial cells were spun at 13000rpm for 5 minutes, supernatant removed and the pellet was re-suspended in 250µl P1 buffer. 250µl P2 buffer was added and mixed thoroughly. 350µl N3 buffer was added, mixed by inversion and centrifuged at 13000rpm for 10 minutes. The supernatant was transferred into QIAspin columns, centrifuged for 1 minute after which the flow through was discarded. 750µl of PE buffer was added to the column and centrifuged at 13000rpm for 1 minute, supernatant was discarded and the plasmid was eluted with 30µl of analytical grade water by centrifugation for 1 minute at 13000rpm. All buffers described above are from the Qiagen mini prep kit.

SW1353 transfection

Chondrosarcoma cell line SW1353 cells were plated at 4 x 10^4 cells/ml (500µl/well in a 24 well plate) and incubated overnight at 37°C. Cells were transfected with 500ng Lipofectamine 2000, 200ng of CAGA plasmid and 50ng Renilla plasmid overnight (Jonk, Itoh et al. 1998). SW1353 cells were serum starved overnight (DMEM alone). SW1353 cells were chosen as they are a fast growing cell line that are easily transfected.

Luciferase assay

SW1353 cells transfected with CAGA and Renilla constructs were incubated for 6 hours with 500µl conditioned medium from 48 hour strained/non-strained cultures. Duplicate samples of conditioned medium were heated for 5 minutes at 80°C to activate TGFβ before incubation with SW1353 cells. Unconditioned medium ±1ng/ml recombinant TGFβ1 were used as negative and positive controls. The Promega Dual Luciferase™ Reporter Assay was used to measure luciferase activity according to manufacturer's instructions. In brief, SW1353 cells were washed in PBS, treated with 50µl of lysis buffer, scraped and

incubated for 15 minutes at room temperature. 10µl was transferred to a 96 well plate and 50µl of luciferase assay reagent II was added. Luminescence was measured using a Perkin Elmer spectrophotometer (Luminescence 700 setting on the Envision plate reader, 560nm), 50µl of stop and go solution was added and the plate was measured for a second time to measure renilla activity (Luminescence 700 setting on the envision plate reader, 560nm). CAGA luciferase units were normalised to renilla luciferase units to account for transfection efficiency (CAGA Luciferase units/Renilla luciferase units). These values were normalised to the non-conditioned medium and expressed as a percentage of the total TGFβ in non-strained controls.

2.4.2 Western Blotting

TCA precipitation

In order to concentrate medium samples for the detection of metalloproteinase expression using western blotting, Trichloroacetic acid (TCA) precipitation was used. Culture medium was mixed with 10% TCA at a ratio of 2:1 and incubated on ice for 1 hour. The samples were centrifuged for 15 minutes at 13000 x g at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol. The supernatant was removed and the pellet was air dried before resuspension in final sample buffer (2% w/v SDS, 2mM β-mercaptoethanol, 4% v/v glycerol, 40mM Tris-HCl (pH 6.8), 0.01% w/v Bromophenol blue). The pH was adjusted using 10M NaOH as required.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted essentially as described by (Shapiro, Vinuela et al. 1967). In brief, 10% v/v Acrylamide, 365mM Tris-HCl (pH 8.8), 0.1% w/v SDS, 0.05% w/v Ammonium persulphate (APS) and 0.04% w/v Tetramethylethylenediamine (TEMED) were combined to create a running gel. This was allowed to polymerise before a stacking gel (4% v/v Acrylamide, 365mM Tris-HCl (pH 8.8), 0.1% w/v SDS, 0.1% w/v APS and 0.04% w/v TEMED) was added and in turn allowed to polymerise with the addition of the comb to created loading wells. The SDS-PAGE apparatus was assembled and running buffer was loaded into the tank (25mM Tris-HCl, 200mM Glycine and 0.1% w/v SDS).

Western Blotting

Western blotting was conducted essentially as described by (Towbin, Staehelin et al. 1979). In brief, conditioned medium and standards were mixed with 5x sample buffer

(10% w/v SDS, 10mM β-mercaptoethanol, 20% v/v glycerol, 0.2M Tris-HCl (pH 6.8), 0.05% w/v Bromophenol blue) at a ratio of 4:1 and incubated at 100 ^{o}C for 5 minutes. Samples were run on an SDS-PAGE gel at 200v for 45 minutes (See protocol above for SDS-PAGE setup). Proteins were transferred onto PVDF (Polyvinylidene Fluoride membrane) using the semi-dry blot technique in the presence of transfer buffer (0.29% w/v Glycine, 0.58% w/v Tris, 0.037% w/v SDS and 20% w/v Methanol) at 10v for 30 minutes. PVDF membranes were incubated in blocking solution (5% milk powder, Tris Buffered Saline solution [TBS- 150mM NaCl, 100mM Tris, pH 7.5]) for at least 1 hour, rinsed in wash buffer (TBS, 0.1% w/v Tween 20 and 1% w/v milk powder) and incubated in the primary antibody (diluted into antibody buffer; TBS, 0.1% v/v Tween 20 and 2.5% w/v milk, for details of primary antibodies used see table 2.4) for 1 hour. The PVDF's were rinsed and incubated (3 x 5 minutes) in wash buffer and incubated in secondary antibody (1:4000 dilution, for details of secondary antibodies see table 2.4) for 30 minutes. The PVDF was rinsed and incubated (3 x 5 minutes) in wash buffer and rinsed and then incubated for 40 minutes in CDP star buffer (0.1M Tris HCl and 0.1M NaCl at pH 9.5). CDP star reagent (chemilluminescent substrate) (diluted in CDP star buffer 1:4000) was added to the protein saturated side of the PVDF. The PVDF was then incubated with chemilluminescent film which was developed using the Odyssey Xograph (LI-COR Biosciences UK Ltd, Cambridge).

Protein of interest	Primary Antibody	Secondary Antibody
MMP1	Rabbit polyclonal TCS Cellworks (1:400 dilution)	Goat polyclonal anti-Rabbit IgG, Alkaline Phosphatase, Abcam ab6722-1, (1:1000 dilution)
MMP2	Mouse monoclonal TCS Cellworks (1:400 dilution)	Rabbit polyclonal to anti-Mouse IgG, Alkaline Phosphatase. Abcam ab6729-1, (1:1000 dilution)
MMP13	Mouse monoclonal TCS Cellworks (1:4000 dilution)	Rabbit polyclonal to anti-Mouse IgG, Alkaline Phosphatase. Abcam ab6729-1 (1:1000 dilution)
MMP23	Rabbit polyclonal TCS Cellworks (1:250 dilution)	Goat R&D systems anti-Rabbit IgG, Alkaline Phosphatase, Abcam ab6722-1, (1:1000 dilution)
ADAM12	Goat polyclonal Abcam ab28747-100	Rabbit polyclonal to anti-Goat IgG, Alkaline Phosphatase. Abcam ab6742-1, (1:10000 dilution)
All isoforms of TGFβ	Pan Specific polyclonal antibody NB-100-NA Rabbit IgG (2µl/ml)	Goat polyclonal anti-Rabbit IgG, Alkaline phosphatise, Abcam ab6722-1, (1:1000 dilution)
TGFβ1	R&D systems MAB240 Mouse Monoclonal IgG (2µl/ml)	Rabbit polyclonal anti-mouse IgG, Alkaline Phosphatase, Abcam ab67291, (1:1000 dilution)
TGFβ2	R&D systems AB-122-NA Goat IgG (2µl/ml)	Rabbit anti-Goat IgG, Alkaline Phosphatase. Sigma A4062. (1:10000 dilution)
TGFβ3	R&D systems MAB243 Mouse IgG (2µl/ml)	Rabbit polyclonal anti-mouse IgG, Alkaline Phosphatase, Abcam ab67291, (1:1000 dilution)

Table 2.4: Western blot antibodies Suppliers: R&D systems (Abingdon), Abcam (Cambridge), Sigma Aldrich (Dorset), TCS Cellworks (Buckingham)

2.4.3 TGFβ ELISA

Freeze drying

In order to concentrate medium samples for the detection of TGFβ expression using ELISA, the freeze drying protocol was used. 500µl of conditioned medium was aliquoted into a vented micro-centrifuge tube (a small hole was made in the lid with a syringe needle), frozen to at -80°C overnight before it was placed in the desiccation chamber of the freeze drying apparatus (Edwards, Crawley, West Sussex). A vacuum was set to remove moisture from the samples in the desiccation chamber for a period of 8 hours. After 8 hours the samples were dry. The protein pellets were resuspended in 100µl of analytical grade water.

Enzyme Linked Immunosorbent Assay (ELISA)

Conditioned medium was freeze dried and re-suspended in analytical grade water (5x concentration). Medsorp microplates (Fisher, Leicestershire) were incubated overnight at 4°C with anti-TGFβ1 capture antibody (Purified rat anti-mouse, human, pig, 4µg/ml) in binding solution (0.1M Na HPO_4, Adjust to pH9 with 0.1M NaH_2P0_4). TGFβ1 antibody coated plates were blocked (10% w/v BSA, 0.1% w/v tween in PBS) for 1 hour and rinsed with wash buffer (0.1% v/v Tween 20 in phosphate buffered saline [PBS]). Samples and standards were incubated on the plate overnight at 4°C and the plate was rinsed in wash buffer. Biotinylated anti-TGFβ detection antibody (2µg/ml) diluted in blocking buffer was incubated on the plate for 1 hour at room temperature. The plate was washed in wash buffer and incubated in Avidin-HRP (Chemilluminescent substrate) diluted 1:1000 in blocking buffer plus Tween 20 (10% BSA, 0.1% tween in PBS). The plate was washed and incubated in TMB (Tetramethylbenzidine) for 30 minutes before the reaction was stopped by adding 1M Sulphuric acid (colour change to yellow). The absorbance was read on a Perkin Elmer spectrophotometer at 450nm (and corrected to the background of 550nm or 630nm). Data was either presented as relative value or percentage of total TGFβ in control samples. Recombinant TGFβ1 (R&D systems, Abingdon) was used to create a standard curve (0.1, 0.2, 0.5, 1 and 5ng/ml). An estimate of the levels of TGFβ in the medium was calculated using the standard curve (exponential trend line).

2.4.4 Gelatin Zymography

Gelatin Zymography was conducted essentially as described in (Clark, Young et al. 2010). In brief 10% resolving SDS-PAGE gel containing gelatin was prepared and incubated for 1 hour at room temperature (1% w/v gelatin, 0.6M TRIS, 10% v/v Acrylamide, 0.1% w/v Sodium Dodecyl Sulphate [SDS], 0.1% w/v ammonium persulphate [APS] and 0.04% v/v TEMED). A 5% stacking gel (0.12M TRIS, 5% v/v Acrylamide, 0.1% w/v SDS, 0.1% w/v APS and 0.04% v/v TEMED) was loaded above the resolving gel and allowed to set for 30 minutes at room temperature. Conditioned medium and loading buffer (200mM Tris pH 6.8, 4% w/v SDS, 0.1% w/v Bromophenol blue and 40% w/v Glycerol) was loaded onto the SDS-PAGE gel (containing gelatin) as well as a positive control (10ng MMP2 and MMP9) and run at 150V for 90 minutes. Gelatin gels were incubated twice for 15 minutes in rinse buffer (5% Triton-X100) to remove SDS and incubated overnight in incubation buffer (50mM Tris pH 7.5 and 5mM CaCl2)(+ ~2ml rinse buffer remaining) at 37^{o}C, rinsed with water and stained in Coomassie Brilliant Blue (30% v/v Isopropanol, 10% v/v Acetic acid and 2.5mg/ml Coomassie Brilliant Blue R) overnight. Gels were rinsed in water, incubated in destain (10% v/v isopropanol and 10% v/v acetic acid) for 1 hour and rinsed in water before imaging using the Licor Odyssey imaging system. Quantification of each band was achieved using image J software. As the standard (10ng of recombinant MMP2) appeared over exposed relative quantification was calculated by normalising these data to the 48 hour proMMP2 band.

2.4.5 QF24 assay; Metalloproteinase activity assay

QF24 is a quenched-fluorescence substrate that can be cleaved by MMPs [Mca-ProLeu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Uria and Lopez-Otin 2000)]. Cleavage of QF24 increases the level of fluorescence emitted by the substrate. Therefore an increase in MMP activity correlates with an increase in fluorescence. Conditioned medium from SW1353 cells stimulated with IL1 was collected as this medium contains a high level of active MMPs; this was used as a positive control. 5µl of bovine nasal cartilage conditioned medium was aliquoted into each well of a white 96 well plate, to selected wells 1.6µM EDTA was added as a negative control. 5µl of conditioned medium from samples treated ±GM6001 or inactive control for 24 and 48 hours was added to selected wells to test the ability of the

GM6001 to inhibit MMP activity in the bovine nasal cartilage conditioned medium. To all wells FAB buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 10mM $CaCl_2$ and 0.05% Brij 35) (each well was made up to 200µl final volume) and 1µM QF24 fluorescent substrate was added. The fluorescence was measured using a Perkin Elmer spectrophotometer to measure the level of MMP activity (excitation wavelength of 328 nm, emission wavelength of 393 nm).

2.4.6 Immunostaining of tenocyte seeded collagen gels

Cell seeded collagen gels were harvested after 48 hours of strain. Gels were immersed in Cryo-M-Bed embedding compound, frozen for 1 hour at -20°C followed and overnight at -80°C. 15µm cryosections were cut using a cryostat (Jencons, Bedfordshire) at -30°C. Sections were mounted onto 3-triethoxysilylpropylamine (TESPA) coated slides (see protocol below) and incubated at -20C overnight. Sections were fixed in 4% paraformaldehyde for 1 hour, washed in PBS and incubated for 30 minutes in 0.5% Triton-X100. Sections were washed in PBS, blocked overnight (in 1% w/v BSA in PBS) at 4°C, washed in PBS, incubated overnight in primary antibody (for details of primary antibodies used see table 2.5) (in PBS), washed in PBS and blocked overnight at 4°C. Sections were washed in PBS, incubated in appropriate secondary antibody (see table 2.5 for details of secondary antibodies used) for 2 hours, washed in PBS and blocked for a further 2 hours in (0.1% w/v BSA in PBS). Blocking solution was removed using a PBS wash and Vectasheild (Fisher, Leicestershire) fluorescence marker imaging fluid containing 4',6-diamidino-2-phenylindole (DAPI) was added to the sections before a cover slip was mounted. DAPI is a fluorescent stain that is absorbed by the nucleus of the cell, this/ allowed us ascertain whether the proteins detected by the antibody were localised to the nuclei. Sections were imaged using a charge-coupled device (CCD) upright Zeiss fluorescence microscope with an attached digital camera and Axiovision 4.7 software (Zeiss, Oberkochen, Germany).

TESPA coating of microscope slides

Glass slides were immersed in acetone for 30 seconds then in acetone plus 5% v/v 3-triethoxysilylpropylamine (TESPA) for 1 minute. Slides were then immersed in acetone twice before a final rinse in distilled water. Slides were dried at 37°C before use.

2.4.7 Statistical analysis

Data was presented as a mean ± standard error (or where n=1 data are shown as the mean ± standard deviation). Data was tested for normal distribution using the Shapiro-Wilk normality test. The Wilcoxon signed rank test and the Students T test (2 tailed, assuming unequal variance) (the paired T test is used where appropriate) were used to analyse qRT-PCR data and TGFβ luciferase data using SASW Statistics 18 and Microsoft Excel as appropriate. $p<0.05$ was chosen as the cut off for statistical significance.

Protein Target	Primary Antibody	Secondary Antibody
TGFβ1	Anti-TGFβ1, Rabbit Polyclonal, Santa Cruz Biotechnology sc-146 (1:200 dilution)	Goat polyclonal antibody to Rabbit IgG (Cy3 labelled) Abcam, ab6939 (1:40 dilution)
TGFβRI	Anit-TGFβRI, Rabbit polyclonal, Santa Cruz Biotechnology sc-398 (1:200 dilution)	
SMAD2	Anti-SMAD2, Rabbit monoclonal IgG Cell Signalling 3122. (1:400 dilution)	
Phospho-SMAD2	Anti-phospho-SMAD2 (Ser465/467), Rabbit monoclonal IgG Cell signalling 3108, , (1:500 dilution)	

Table 2.5: Immunostaining antibodies Suppliers: Cell signalling (Hertfordshire), Santa Cruz Biotechnology (Insight Biotechnology Ltd, Middlesex), Abcam (Cambridge)

Chapter 3: Optimisation

3.1. CELL VIABILITY TESTING

In order to validate our experiments we needed to confirm that the cells were viable in the 3D collagen gels in the presence or absence of mechanical load. To test the viability of cells following a loading regime of 0-5% cyclic strain at 1Hz for a period of 48 hours we used two methods of viability testing: Calcein AM (Live cells) and ethidium homodimer (dead cells) staining and collagenase extraction of cells, followed by quantification of dead cells using trypan blue staining.

3.1.1. Methods

Calcein AM and Ethidium Homodimer staining

Tenocyte-seeded 3D gels were stained using Calcein AM, which localises to the cytoplasm of viable cells and Ethidium homodimer, which binds to DNA in the nucleus of dead cells. In brief, 3D collagen gels were washed in PBS and incubated in phenol-free DMEM containing both Calcein AM and ethidium homodimer (1/20 dilution) for 45 minutes at 37°C. Gels were transferred to slides and cover slips were mounted for viewing on the charge-coupled device (CCD) upright Zeiss fluorescence microscope with an attached digital camera and Axiovision 4.7 software (Zeiss, Oberkochen, Germany). Calcein AM fluoresces green (500-530) when excited with blue light (488 nm) and ethidium homodimer emits a red fluorescence (600-650) when excited with green light (564nm).

Tenocyte extraction by collagenase digestion

Tenocyte seeded 3D gels were washed in PBS and digested in collagenase for 5 hours (0.1% Collagenase, 0.4% HEPES in DMEM). Cells were centrifuged at 150 x g for 5 minutes, supernatant was removed and DMEM was added; this process was repeated three times to remove collagenase. Cells were pelleted and re-suspended in a known volume of DMEM. Cells were stained with trypan blue (cells stained blue are non-viable) and

counted using a haemocytometer. Live and dead cells were quantified, and comparisons were made between cells treated ±strain.

3.1.2. Results and Discussion

Calcein AM and Ethidium Homodimer staining

Cell seeded collagen gels exposed to 1 hour at -20°C were used as positive controls for ethidium homodimer staining as this caused 100% cell death. Figure 1 B and D show positive controls where cells are clearly stained with ethidium homodimer at their nucleus, confirming that the dead cell staining was successful. In addition in these positive controls no calcium AM staining was evident indicating that the live cell staining is specific. In non-strained and strained samples there was high levels of calcium AM staining indicating that a high percentage of cells were viable, in addition there was little ethidium homodimer staining indicating low levels of cell death. There appeared to be less cell death in strained cultures compared to non-strained controls; however we were unable to quantitate this due to the compact nature of cells. It was clear that in strained cultures the cells were more tightly packed, making it more difficult for us to quantitate these results. As a result we chose to use an additional means to measure cell viability that enabled us to measure viability in a more quantitative manner. Calcein AM staining of the cytoplasm of live cells also indicates that in strained cultures the cells are aligned to the direction of loading, whereas in non-strained cultures this is much less obvious. This indicates that cells are able to adapt in morphology in response to mechanical load.

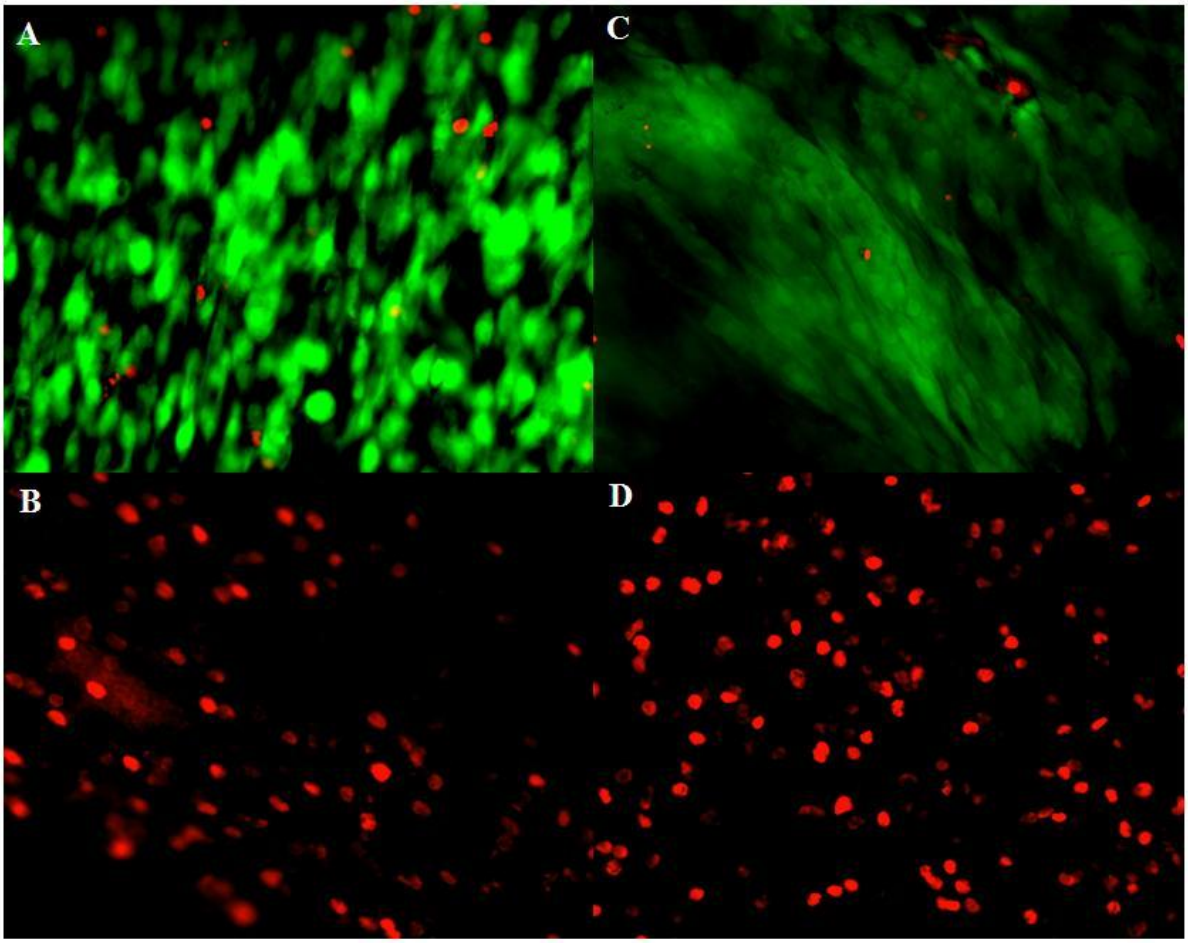

Figure 3.1. Calcein AM and Etidium Homodimer cell viability testing. Primary human Achilles tenocytes were seeded into type I rat tail collagen (final concentration of $1.5x10^6$ cells/ml and 1mg/ml collagen) and cyclically strained (5% at 1Hz) for 48 hours. Cells were stained using Calcein AM (live cells – green) and Ethidium Homodimer (dead cells – red). Non-strained (A and B), strained (C and D) and positive controls for Ethidium homodimer staining (B and D) are shown (frozen for 1 hour at -20°C). These images are representative of a large number of images taken at different positions of the 3D culture (n=1).

Tenocyte extraction by collagenase digestion

Live and dead cells were counted and data expressed as a percentage of the total cell number. The average of three experiments is shown. The number of dead cells was 3% and 4% in non-strained and strained cultures respectively (see figure 3.2); however the number of dead cells was not significantly different between strained and non-strained cultures (T test [2 way, unequal variance]).

There was a high percentage of viable cells and a low level of dead cells in the collagenase digestion and trypan blue staining experiment, consistent with the ethidium homodimer and Calcein AM staining. However, cell numbers were lower than expected when cells were counted following collagenase digestion (~50% of the cells seeded into the gel). This is most likely due to the loss of cells during the centrifugation stages to wash collagenase from the cells. However due to the consistency of low cell death with the Calcein AM and ethidium homodimer staining we can conclude that the level of cell viability is high.

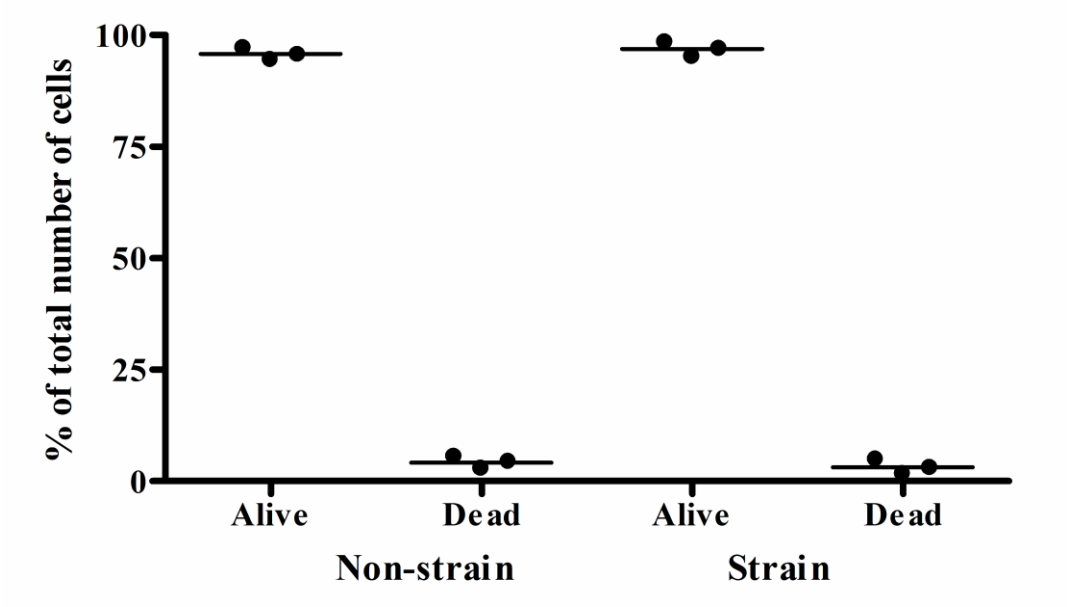

Figure 3.2. Collagenase digestion and trypan blue staining: cell viability testing. Primary human Achilles tenocytes were seeded into type I rat tail collagen (final concentration of $1.5x10^6$ cells/ml and 1mg/ml collagen) and cyclically strained (5% at 1Hz) for 48 hours. Cells were digested in bacterial collagenase and stained with trypan blue (only dead cells take up the trypan blue stain). Both live and dead cells were counted and expressed as a percentage of the total number of cells (n=3).

3.2. TGFβ CONCENTRATION TITRATION

TGFβ expression is increased in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002); therefore we were interested in treating tendon cells with TGFβ to observe the effects of TGFβ in conjunction with mechanical loading. We have shown COL1A1 is stimulated in response to TGFβ; this gene was chosen as a measure of the cells response to TGFβ. Our aim was to determine the optimum concentration of TGFβ required to stimulate a response in COL1A1 expression.

3.2.1. Method

Primary human Achilles tenocytes were trypsinised and re-suspended at $3x10^6$ cells/ml (double the final density) in serum free medium (1% penicillin [10mg/ml] / streptomycin [10u/ml]). Rat tail type I collagen (2.2mg/ml) was mixed with 10x DMEM at a ratio of 9:1 and the pH was adjusted to approximately 7 by the addition of 10M Sodium Hydroxide (NaOH), using DMEM colour (Orange/red colour indicates a pH of approximately 7) to assesss the Ph. Neutralised collagen and tenocyte suspension were mixed 1:1 (1mg/ml collagen, $1.5x10^6$ cells/ml final density) and 200µl was pipetted into each well of a 48 well plate. Gels (plus tenocytes) were allowed to set for 1 hour at 37 ^oC, at which point 500µl serum free DMEM was added (1% penicillin [10mg/ml] / streptomycin [10u/ml]). A range of concentrations of TGFβ (0, 0.1, 0.5, 1, 2, 5, 8 and 10ng/ml) was added to the tenocyte seeded collagen gels. Gels (plus tenocytes) were harvested after 48 hours; medium was removed and gels were dissolved in 4 x the gel volume of trizol. RNA was isolated using the tri-spin protocol, reverse transcribed and standard qRT-PCR was used to analyse TOP1 (endogenous control) and COL1A1. The COL1A1 data was normalised to TOP1 and expressed as $2^{\Delta Ct}$. The Students t-test (two tailed, paired) was used to assess statistical significance, $p<0.05$ was chosen as the cut off for significance.

3.2.2. Results and Discussion

COL1A1 expression was increased with TGFβ (Figure 3). At 0.1, 0.5, 5 and 8ng/ml there was a trend to increase in COL1A1 expression with TGFβ treatment, however this was not significant. With 1, 2 and 10ng/ml TGFβ there was a significant increase in COL1A1 expression. Therefore 1ng/ml was chosen as the most effective and economical concentration of TGFβ.

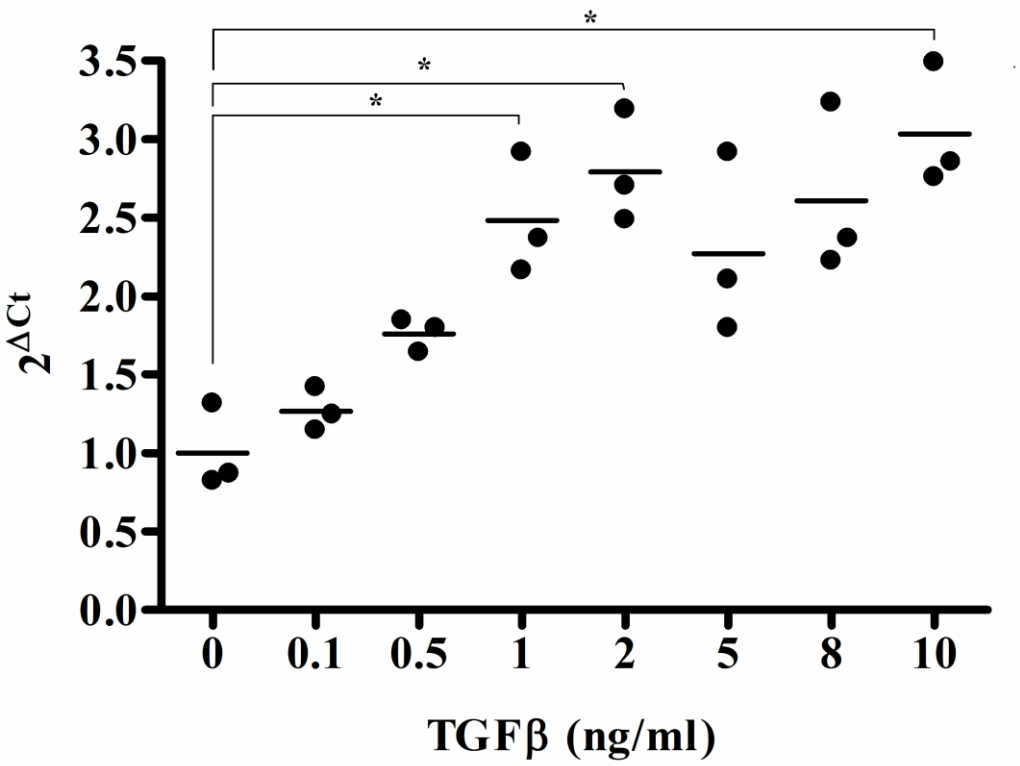

Figure 3.3. COL1A1 expression in response to TGFβ treatment. Primary human Achilles tenocytes were seeded into type I rat tail collagen (final concentration of $1.5x10^6$ cells/ml and 1mg/ml collagen) and treated with a range of TGFβ concentrations (0, 0.1, 0.5, 1, 2, 5, 8 and 10ng/ml). COL1A1 mRNA was measured using qRT-PCR. Data was normalised to TOP1 and expressed as $2^{\Delta Ct}$. The students t test (two tailed, paired) was used to assess statistical significance, $p<0.05$ was chosen as the cut off for significance (n=3).

3.3. TGFβ LUCIFERASE ASSAY SPECIFICITY CONFIRMATION

We used a cell based luciferase assay to measure active TGFβ (See chapter 5). However because this assay was a measure of SMAD activity we needed to confirm that TGFβ signalling and more specifically TGFβ activity is responsible for the stimulation of SMAD activity. In order to test this we incubated strain and non-strained conditioned medium with either the TGFβRI inhibitor or a Pan-TGFβ inhibitory antibody in the cell based assay. In addition we used unconditioned medium ±TGFβ (5ng/ml) in combination with either the TGFβRI inhibitor or a Pan-TGFβ inhibitory antibody in the cell based assay. This would allow us to test the TGFβ inhibitory ability of these inhibitors as well as to confirm that the soluble mediator of downstream SMAD activation was TGFβ.

In order to test which isoform was activated in response to mechanical load (See chapter 5, section 5.3.6), functionally inhibiting antibodies targeting TGFβ isoforms 1, 2 and 3 were added to the luciferase assay with strain and non-strain conditioned medium. TGFβ antibodies were also added in combination, i.e. TGFβ1 plus TGFβ3, to determine whether a combination of isoforms was responsible.

3.3.3. Methods

Conditioned medium was collected from tenocyte seeded collagen gels strained for 48 hours at 5% cyclic strain at 1Hz. SWI353 cells transfected with CAGA and Renilla constructs were incubated with strain conditioned medium with the addition of TGFβRI (SB431542, 10μM), TGFβ1 (MAB240, 1-2.5μg/ml), TGFβ2 (AB-122-NA, 1-2.5μg/ml), TGFβ3 (MAB243, 1-2.5μg/ml) or Pan-TGFβ inhibitory antibody (2.5μg/ml) for 6 hours (TGFβ isoforms were also added in combination). In addition unconditioned medium ±TGFβ (5ng/ml) was incubated with TGFβRI (SB431542, 10μM) or Pan-TGFβ inhibitory antibody (2.5μg/ml) with the transfected SW1353 cells for 6 hours. Medium was removed and cells were washed in PBS before cells were lysed for 15 minutes. Cell lysate was transferred into a 96 well plate (10μl) to which 50μl of luciferin was added (Dual luciferase reporter assay). The absorbance was read on a spectrometer (CAGA), stop and glo solution (from the dual luciferase reporter assay kit) was added and the plate was read again (Renilla) (Dual luciferase reporter assay). CAGA absorbance units (measure of SMAD activation) were normalised to the Renilla absorbance units (transfection control). These values where then normalised to negative control samples. For more details on the TGFβ

luciferase assay see methods section. The t test was used to assesss statistical significance (assuming unequal variance and two tailed distribution), $p<0.05$ was chosen for the cut off for significance.

3.3.4. Results and Discussion

TGFβ significantly stimulated SMAD activation compared to samples incubated with non-conditioned medium only (Figure 3.4). Both the TGFβRI inhibitor and the Pan specific TGFβ inhibitory antibody significantly abrogated SMAD activation. Strain conditioned medium showed a significantly high level of SMAD activation compared to non-strained controls. With the addition of the TGFβRI inhibitor the strain induced activation of SMAD was completely abrogated. This suggests that the SMAD is activated via TGFβRI by a soluble factor present in the conditioned medium of strained Achilles tenocytes. With the addition of the Pan specific TGFβ inhibitory antibody there was a significant reduction in the strain conditioned medium stimulated response. As the Pan specific TGFβ antibody is thought to inhibit the activity of all TGFβ isoforms, this indicates that TGFβ is the soluble factor present in strain conditioned medium that stimulates SMAD activation. Antibodies more specific to the isoforms of TGFβ could therefore isolate the predominant isoform or isoforms activated in response to mechanical load. However, none of the antibodies we tried successfully abrogated the strain induced SMAD activation, despite the use of these antibodies in combination (data not shown). This is likely due to the ineffective nature of the antibodies tested.

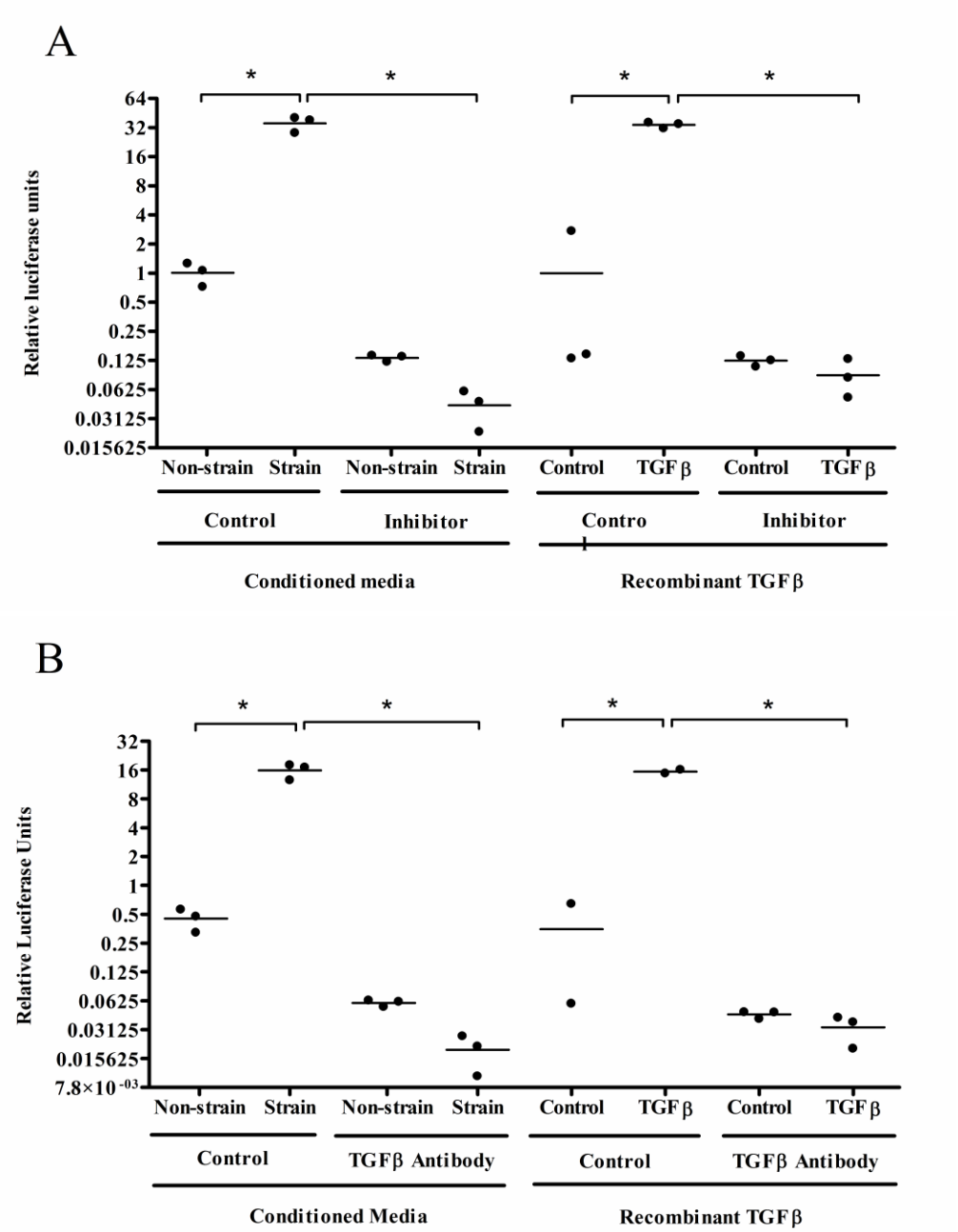

Figure 3.4. TGFβ luciferase assay specificity test. The TGFβ luciferase assay gives a reading of relative luciferase units that is a measure of SMAD activity. In order to confirm that TGFβ signalling and more specifically TGFβ activity is responsible for the stimulation of SMAD activity, inhibitors of TGFβ activity and TGFβ signalling were used. Non-conditioned media ± TGFβ, strain and non-strain conditioned media was incubated with TGFβRI inhibitor (A) and a Pan specific TGFβ inhibitory antibody (B) to confirm the measure of SMAD activation was stimulated via TGFβ. Statistical analysis was performed using the t test (assuming unequal variance and two tailed distribution) and the statistical cut off was chosen as $p<0.05$ (n=3, conditioned media from one cell isolate only).

3.4. GM6001 ACTIVITY TEST

As GM6001 had no effect upon the strain mediated activation of TGFβ or metalloproteinase gene expression (presented section 6.3.1.), we needed to confirm that GM6001 added to cultures was persistently active after 8, 24 and 48 hours of culture with primary Achilles tenocytes. A fluorescent MMP substrate called QF24 was used to assesss the inhibitory activity of GM6001 in the presence of conditioned medium with high levels of active MMPs.

3.4.1. Method

Conditioned medium from cultures of the bovine nasal cartilage treated with IL1 for 14 days contains a high level of active MMPs. 5µl of bovine nasal cartilage conditioned medium was aliquoted into each well of a white 96 well plate, to selected wells EDTA (1.6µM final concentration) was added as a negative control (inhibitor of MMP activity). 5µl of strain or non-strained conditioned medium from samples treated ±GM6001 or inactive control (similar peptide structure to GM6001 that does not inhibit metalloproteinase activity) for 24 and 48 hours was added to selected wells to test the ability of the GM6001 to inhibit MMP activity in the bovine nasal cartilage conditioned medium (positive control). To all wells FAB buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 10mM $CaCl_2$ and 0.05% Brij 35) (each well was made up to 200µl final volume) and QF24 (1µM final concentration) fluorescent substrate was added. The absorbance was measured using a perk and Elmer spectrophotometer to gauge the level of MMP activity (excitation wavelength of 328 nm, emission wavelength of 393 nm).

3.4.2. Results and Discussion

Conditioned medium from bovine nasal cartilage (positive control) caused an increase in the level of absorbance units which peaked at approximately 1000000 units after 61 readings (Figure 5); this indicates that the high level of MMP activity caused the degradation of the QF24 substrate. Where a combination of positive control medium and EDTA was assessed, there was a reduction in the level of absorbance units after 61 readings (200000units) compared to positive control medium alone. EDTA prevents MMP activity therefore degradation of QF24 is completely abrogated. A combination of GM6001 (250µM) and positive control medium also caused a decrease in the level of MMP activity, compared to the positive control. However, this reduction was not as

marked as samples incubated with EDTA. A combination of positive control medium and conditioned medium (±strain) with the addition of GM6001 showed a decrease in relative absorbance units after reading 61 compared to positive control. There was no clear difference ±strain or between different time points. This indicates that the GM6001 added to these samples at the start of culture was still active after 48 hours of culture. There was little difference between positive control cultures and a combination of strain conditioned medium with inactive GM6001 and positive control medium in terms of relative absorbance units, this indicates that inactive GM6001 has no effect upon MMP activity, as expected.

Failure to use an equal amount of GM6001 in the negative control as added to the cultures means that cannot confirm that GM6001 retained its full activity during its 48 hour culture period (0.1μM in the QF24 assay from conditioned medium samples, 250μM was used in the QF24 assay as a negative control). However we can still see that GM6001 retained some activity in the conditioned medium. And as the positive control medium contained far more MMP activity that in the tenocyte culture medium (MMP activity from the tenocyte conditioned medium was undetectable, data not shown), we can assume that this was sufficient to inhibit any metalloproteinase activity after 48 hours of culture.

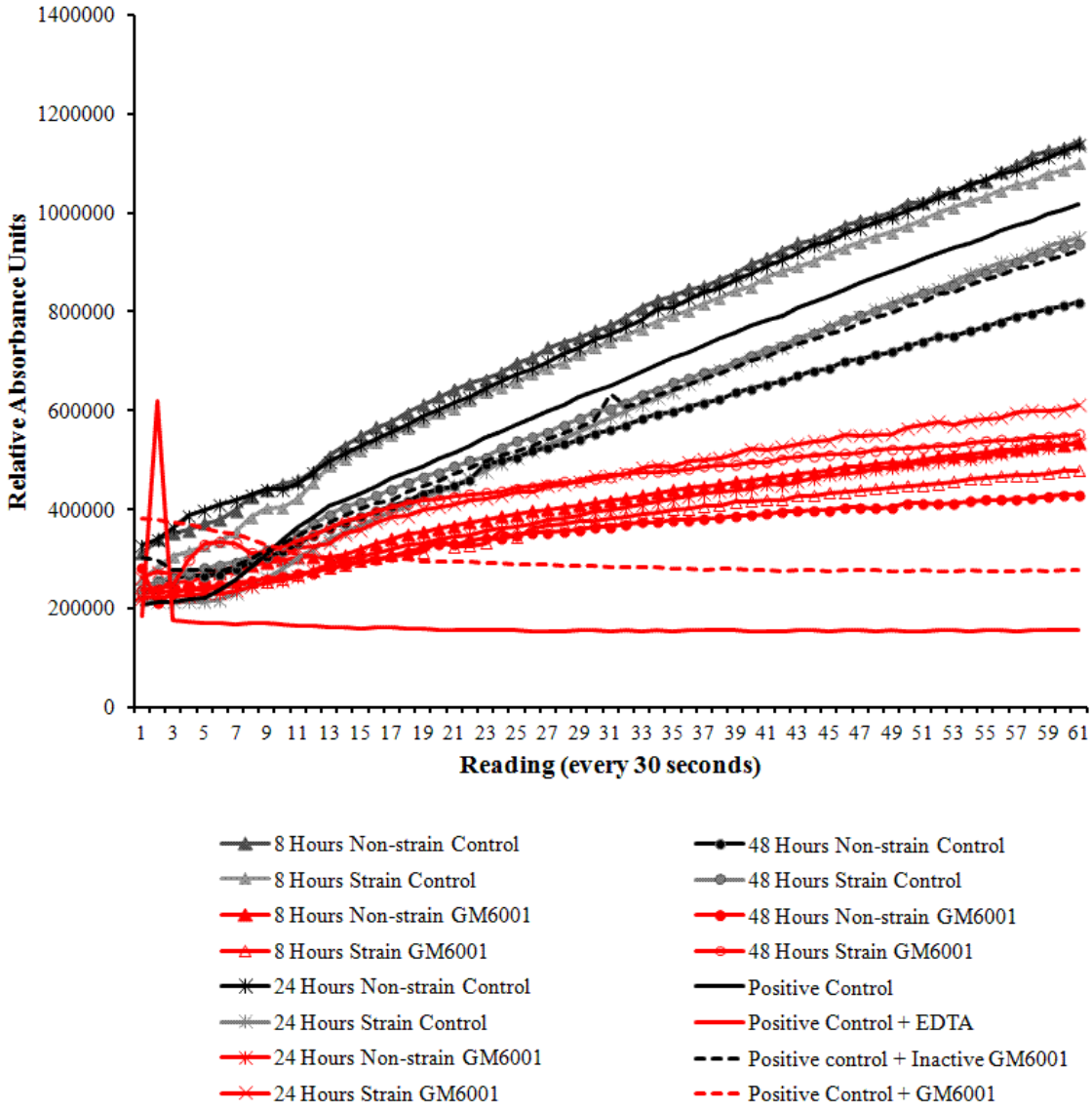

Figure 3.5. A fluorescent MMP substrate (QF24) as a measure the inhibitory activity of GM6001. Conditioned media was collected from tenocyte seeded type I rat tail collagen gels strained at 5% at 1Hz for 8, 24 and 48 hours, ±GM6001 / Control peptide. Conditioned media from cartilage explants cultured for 14 days was used as a positive control due to the high level of MMP activity. Positive control conditioned media was used in all conditions in the presence or absence of tenocyte conditioned media. EDTA, GM6001 and inactive GM6001 were added as additional controls in the absence of strain conditioned media. Samples with red markers are those containing GM6001 or EDTA control. Those in black contain ± control inactive GM6001 (n=1).

3.5. TGFβ LUCIFERASE ASSAY REAGENT INTERFERENCE TEST

As the TGFβ luciferase is a cell based system, there is a risk that cell treatments may affect the relative measure of TGFβ activity. To test whether treatments (see chapter 7) affected this measure we examined their effects upon TGFβ stimulation of the cell based luciferase assay.

3.5.1 Methods

Apyrase (10U/ml), M6P (10μM), SB203580 (5μM), Doramapimod (0.1μM), PD0325901 (0.1μM), U-0126 (20μM), Wortmannin (0.1μM) and PI-103 (0.5μM), were added (along with TGFβ and non-conditioned DMEM) to the CAGA and Renilla transfected SW1353 cells and incubated for 6 hours. Medium was removed and cells were washed in PBS before cells were lysed for 15 minutes. Cell lysate was transferred into a 96 well plate (10μl) to which 50μl of luciferin was added (Dual luciferase reporter assay). The absorbance was read on a spectrometer (CAGA), stop and glo solution was added and the plate was read again (Renilla) (Dual luciferase reporter assay). CAGA absorbance units (measure of SMAD activation) were normalised to the Renilla absorbance units (transfection control). These values where then normalised to negative control samples. For more details on the TGFβ luciferase assay see methods section.

3.5.2. Results and Discussion

TGFβ treatment induced a significant increase in the level of active TGFβ detected using the TGFβ luciferase assay compared to control. In TGFβ treated cultures M6P, Doramapimod, PD0325901, U-0126 and Wortmannin significantly increased the level of active TGFβ detected in the luciferase assay compared to controls (without TGFβ treatment). However, Apyrase, SB203580 and PI-103 did not show a significant level of active TGFβ in TGFβ treated cultures according to the luciferase assay. In TGFβ treated cultures Apyrase, M6P, U0126 and PI-103 treatment showed significantly less detection of active TGFβ compared to TGFβ treated controls. Therefore, Apyrase, M6P, U0126 and PI-103 significantly affected the detection of TGFβ using the cell based luciferase assay, this means that conditioned medium from cells initially treated with these inhibitors may not give an accurate reading in terms of TGFβ when analysed using the luciferase assay. Here we show the effect of fresh inhibitor directly on the luciferase assay, as the treatments are

incubated at 37°C for a period of up to 48 hours before the luciferase assay, in the full experiment their ability to interfere with the luciferase assay may have decreased. Therefore these results may show an exaggerated level of inhibition of the luciferase assay. In order to confirm this, we could test treatments incubated at 37°C for 48 hours.

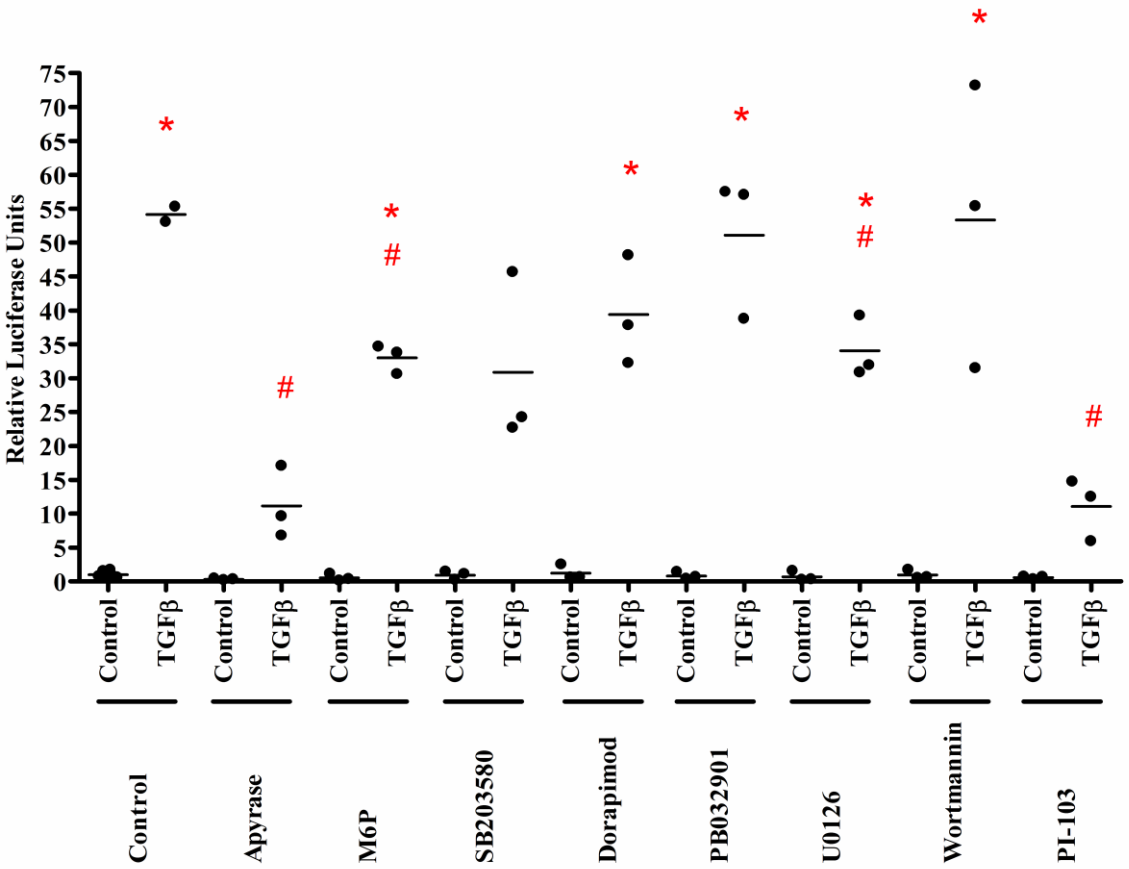

Figure 3.6. TGFβ luciferase assay reagent interference test. Apyrase (10U/ml), M6P (10µM), SB203580 (5µM), Doramapimod (0.1µM), PD0325901 (0.1µM), U-0126 (20µM), Wortmannin (0.1µM) and PI-103 (0.5µM), were added (along with TGFβ and non-conditioned) to the CAGA and Renilla transfected SW1353 cells. The dual luciferase reporter assay was used to assesss the level of active TGFβ. CAGA luciferase units were normalised to renilla luciferase units (transfection control) before being normalised to negative controls. Statistical analysis was performed using the t test (assuming unequal variance and two tailed distribution) and the statistical cut off was chosen as $p<0.05$ (n=3, conditioned media from one cell isolate only). * indicates that there is a significant difference between TGFβ treatment and controls, # indicates a significant difference between the treatment plus TGFβ and controls plus TGFβ.

CHAPTER 4: Mechanical load regulation of metalloproteinase and matrix gene expression

4.1. INTRODUCTION

Mechanical loading across the tendon tissue has been implicated in the development of tendinopathy; however the mechanism is not fully understood. Differential regulation of matrix proteins and metalloproteinase enzymes has been associated with tendinopathy (Ireland, Harrall et al. 2001; Fu, Chan et al. 2002; Riley, Curry et al. 2002; Alfredson, Lorentzon et al. 2003; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006; Clegg, Strassburg et al. 2007; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012) and also with mechanical loading (Archambault, Tsuzaki et al. 2002; Archambault, Elfervig-Wall et al. 2002; Tsuzaki, Bynum et al. 2003; Koskinen, Heinemeier et al. 2004; Yang, Crawford et al. 2004; Screen, Shelton et al. 2005; Yang, Im et al. 2005; Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007; Arnoczky, Lavagnino et al. 2008; Asundi and Rempel 2008; Gardner, Arnoczky et al. 2008; Smith, Sakurai et al. 2008; Sun, Li et al. 2008; Maeda, Shelton et al. 2009; Szczodry, Zhang et al. 2009; Maeda, Fleischmann et al. 2010; Sun, Andarawis-Puri et al. 2010). Therefore we are interested in studying the effects of mechanical loading upon metalloproteinase expression. By further understanding this pathway of mechanical regulation we may begin to understand the role of mechanical load in tendinopathy.

The majority of studies of the regulation of metalloproteinase and matrix genes in response to mechanical load in tendon mainly concern rodent tissue or cells, with a shortage of human studies (Archambault, Tsuzaki et al. 2002; Archambault, Elfervig-Wall et al. 2002; Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004; Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007; Arnoczky, Lavagnino et al. 2008; Asundi and

Rempel 2008; Gardner, Arnoczky et al. 2008; Leigh, Abreu et al. 2008; Sun, Li et al. 2008; Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010; Sun, Andarawis-Puri et al. 2010). In addition, the majority of these studies have looked at only a very limited array of metalloproteinase and matrix genes, mainly focussing on MMP1, MMP2, MMP3, MMP13 and COL1A1 (Archambault, Tsuzaki et al. 2002; Archambault, Elfervig-Wall et al. 2002; Tsuzaki, Bynum et al. 2003; Koskinen, Heinemeier et al. 2004; Yang, Crawford et al. 2004; Screen, Shelton et al. 2005; Yang, Im et al. 2005; Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007; Arnoczky, Lavagnino et al. 2008; Asundi and Rempel 2008; Gardner, Arnoczky et al. 2008; Smith, Sakurai et al. 2008; Sun, Li et al. 2008; Maeda, Shelton et al. 2009; Szczodry, Zhang et al. 2009; Maeda, Fleischmann et al. 2010; Sun, Andarawis-Puri et al. 2010). The aims of this study include the confirmation of existing evidence that shows MMP1, MMP13 and COL1A1 regulation with mechanical load and to look at a wider range of metalloproteinase, matrix genes, cytokines and transcription factors. We chose these genes on the basis of their involvement in matrix turnover and their regulation in tendinopathy. We hypothesise that there will be a response to loading in a wide range of metalloproteinase, TIMPs and matrix genes.

Tenocytes themselves are not exposed to the same mechanical strain as the whole tendon fascicle due to: fibre alignment, muscle rotation, straightening of the crimp and fibril elongation (Purslow, Wess et al. 1998; Puxkandl, Zizak et al. 2002; Riley 2004; Screen, Lee et al. 2004). The level of strain transmitted to tenocytes ex vivo in tendon fascicles is <2% (Screen, Lee et al. 2004). Screen et al reported that rat tendon fascicles were elongated to between 6-13% in rat tail tendon fascicles cause irreversible damage (Screen, Lee et al. 2004). Therefore this is equivalent to 0.12-0.26% elongation of the cells in the fascicle. One issue about some of the existing studies of tendon mechanical loading is that the strain used is larger than the predicted level of physiologically relevant strain; in vitro cell studies using strain levels as high as 8% strain which is much greater than in tissue studies where irreversible damage has been observed (Lavagnino, Arnoczky et al. 2003; Screen, Lee et al. 2004). For example Wang et al seeded cells directly on a silicone membrane before exposing them to 4 and 8% elongation (Yang, Im et al. 2005), as the cells are in direct contact with the silicone membrane they are exposed to this load in its entirety.

Here we are interested in investigating moderately high levels of physiologically relevant strain; therefore we have chosen 5% strain. Although it is difficult to determine the actual

load that human tenocytes undergo within the tendon matrix due to lack of human studies in this area, this is our best educated guess, taking into account data from rat tendon studies described above. One of the aims of this study is to determine the effects of moderately high mechanical load upon tenocytes and to measure these effects by mRNA analysis of metalloproteinase and matrix genes.

Previous studies have shown that cell-cell contact may play an important role in the cells ability to perceive mechanical load (Wall and Banes 2005). As we have discussed in the introduction, GAP junctions and integrins form links between cells and between cells and their environment. Evidence in the literature supports the role of these molecules in the perception of load (Arnoczky, Lavagnino et al. 2008). Here we aimed to compare two cell densities, a lower and a higher cell density; the latter would show increased cellular proximity and therefore increased cellular interaction, with the opposite effect in low cell density cultures. We hypothesise that a decreased level of cellular interaction will result in a reduced response to mechanical loading and vice versa.

4.2. METHODS

Analysis of gene expression with mechanical load: Tenocytes derived from tendinopathic, normal or ruptured tendon were seeded in type I collagen into Flexcell™ tissue train plates and loaded at 1Hz at 5% strain for a range of time points; 2, 4, 8, 24 and 48 hours at either 0.5 x 10^6 or 1.5 x 10^6 cells/ml. Gels were dissolved in Trizol and RNA was extracted using the tri-spin method essentially as described elsewhere (Ireland, Harrall et al. 2001). RNA was quantitated, reverse transcribed and analysed via qRT-PCR (Taqman) for a range of metalloproteinase and matrix genes (see table 2.1 in methods section). Data was normalised to the housekeeping gene TOP1 and expressed as the $2^{\Delta\Delta Ct}$. Statistical analysis was performed using the Wilcoxon signed rank test after a normality test (Shapiro Wilk W) confirmed the distribution was not normal. For full details see methods section 2.2 and 2.3.

Taqman low density array (TLDA) analysis: Due to the more robust response in cultures seeded at the higher cell density tenocytes were seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen (1mg/ml) were plated into Flexcell™ tissue train plates and uniaxially strained at 5% at 1Hz for up to 48 hours (Metalloproteinase and TIMP family analysis focused on 24 and 48 hours, whereas Time course analysis of selected genes looked at a time course of 2, 4, 8, 24 and 48 hours). Gels were harvested in Trizol and RNA extracted using the tri-spin method essentially as described elsewhere (Ireland, Harrall et al. 2001). RNA was quantitated, reverse transcribed and analysed using TLDA qRT-PCR. Metalloproteinase and TIMP family analysis focused 19 ADAMTS genes, all 23 MMP genes and all 4 TIMP genes. Genes were selected for analysis on the time course TLDA on the basis of robust response in the metalloproteinase and TIMP family TLDA (see table 2.2 and 2.3 in methods section for a full list of the genes analysed).

Data was normalised to 18s and expressed as the $2^{\Delta\Delta Ct}$. Statistical analysis was performed using the Student's t test (two tailed distribution, paired t test), as there were only 3 repeats we were unable to confirm whether these data were normally distributed and non-parametric tests are not suitable for n=3. For full details see methods section 2.3.4. For the TLDA analysis TOP1 was not available for analysis. However, when using standard qRT-PCR, there was very little difference in data normalised to TOP1 and 18s.

Gelatin Zymography – MMP2 detection: Conditioned medium was collected and loaded onto gelatin-containing SDS-PAGE gels essentially as described by Clarke et al (Clark, Young et al. 2010). Gels were incubated overnight at 37°C in order to allow MMP activity to degrade the gelatin and stained using Coomassie Brilliant Blue R. Gels were imaged using the Licor Odyssey imaging system. Relative Quantification was calculated using Image J software. Due to the overexposure of recombinant MMP2 samples data was expressed as a percentage of proMMP2 (unprocessed / inactive) at 48 hours non-strain. Statistical analysis was performed using the Student's t test (2 way, paired t test) as we determined these data were normally distributed using the Shapiro Wilk normality test. For full details see methods section 2.4.4.

4.3. RESULTS

4.3.1. Cell-density dependant changes

There were significant decreases in the mRNA levels in MMP1 (3.2 fold), MMP2 (1.5 fold), MMP3 (1.38 fold), MMP13 (2.3 fold) and Fibulin-1 (2.3 fold) in response to 48 hours cyclical mechanical load in collagen gels seeded with 1.5×10^6 cells/ml (4.1). At the lower cell density only MMP13 (1.4 fold) showed significant changes, in which case the response was less marked than in cultures seeded at the higher cell density.

There were significant increases in ADAM12 (2.1 fold), COL1A1 (1.8 fold) and Elastin (3.8 fold) in response to mechanical load at 24 hours in cells seeded at the higher cell density (See figure 4.1). At 48 hours Elastin (12.4 fold) also showed a significant response to load in cells cultures seeded at the higher cell density. No significant changes were seen at the lower cell density.

Generally responses were less variable at the higher cell density compared to those seeded at the low cell density. Some early responses were evident in cultures seeded at the lower cell density that were not present in cultures seeded at the high cell density. MMP2 (1.6 fold) was increased with load at 2 hours and MMP13 was increased with load at both 2 and 4 hours (1.9 fold and 2.2 fold). For a summary table showing statistical p values see table 4.1.

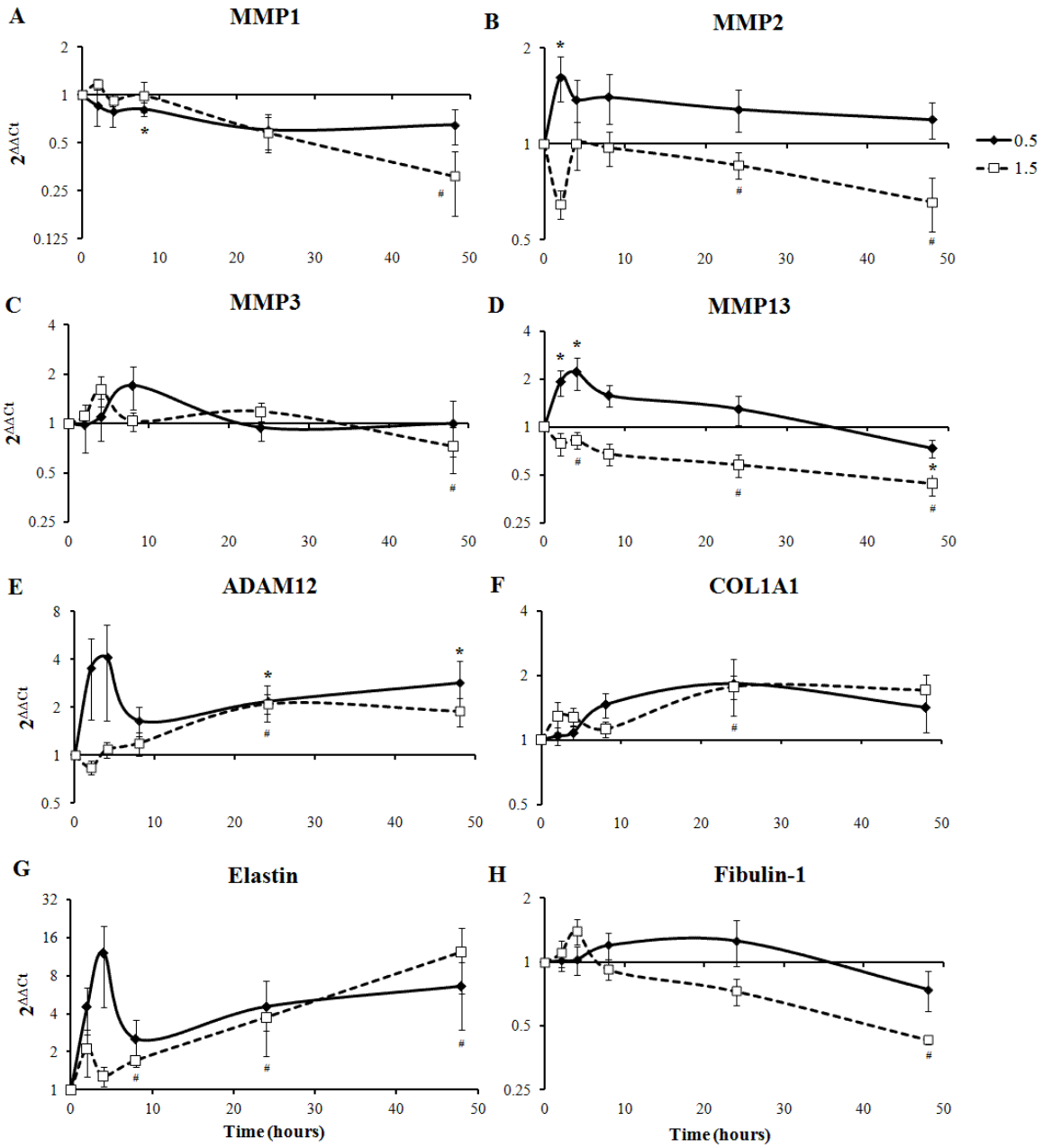

Figure 4.1. Cell-density dependant changes in metalloproteinase and matrix gene expression over a time course. Cells were seeded in type I collagen at either 0.5 or 1.5 x 10^6 cells/ml into Flexcell™ tissue train plates and subjected to 0-48 hours of strain at 5% at 1Hz. mRNA of a number of key metalloproteinase and matrix genes was measured using standard qRT-PCR. Data was normalised to TOP1 and presented as a mean ($n \geq 6$, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). The Wilcoxon signed rank test was used to test statistical significance ($p<0.05$). Significant changes in expression is denoted by * for samples seeded at 0.5 x 10^6 cells/ml or # for samples seeded at 1.5 x 10^6 cells/ml.

A Fold change

$0.5x10^6$ cells/ml

Time (hours)	2	4	8	24	48
MMP1	0.86	0.79	0.82	0.61	0.65
MMP2	1.63	1.38	1.41	1.29	1.19
MMP3	0.98	1.10	1.72	0.94	1.00
MMP13	1.92	2.21	1.58	1.30	0.74
ADAM12	3.54	4.12	1.66	2.18	2.86
COL1A1	1.04	1.08	1.46	1.84	1.42
Elastin	4.55	12.13	2.53	4.55	6.59
Fibulin	1.02	1.03	1.20	1.26	0.74

$1.5x10^6$ cells/ml

Time (hours)	2	4	8	24	48
MMP1	1.17	0.93	1.00	0.58	0.31
MMP2	0.65	1.00	0.97	0.86	0.66
MMP3	1.11	1.61	1.03	1.18	0.72
MMP13	0.79	0.83	0.68	0.58	0.44
ADAM12	0.84	1.09	1.19	2.11	1.90
COL1A1	1.29	1.28	1.12	1.78	1.71
Elastin	2.11	1.29	1.72	3.77	12.38
Fibulin	1.11	1.40	0.93	0.73	0.43

B Statistical significance

$0.5x10^6$ cells/ml

Time (hours)	2	4	8	24	48
MMP1	0.063	0.398	0.028	0.31	0.128
MMP2	0.018	0.128	0.31	0.176	0.398
MMP3	0.398	0.735	1	0.398	0.866
MMP13	0.018	0.018	0.128	0.176	0.043
ADAM12	0.735	0.735	0.176	0.018	0.018
COL1A1	1	0.465	0.068	0.068	1
Elastin	0.128	1	0.237	0.866	0.866
Fibulin	0.735	0.866	0.091	0.499	0.31

$1.5x10^6$ cells/ml

Time (hours)	2	4	8	24	48
MMP1	0.128	0.333	0.333	0.051	0.021
MMP2	0.612	0.445	0.508	0.038	0.015
MMP3	0.237	0.386	0.114	0.953	0.01
MMP13	0.091	0.038	0.086	0.012	0.008
ADAM12	0.735	0.386	0.959	0.008	0.051
COL1A1	0.398	0.091	0.398	0.028	0.173
Elastin	0.463	0.249	0.018	0.046	0.046
Fibulin-1	0.866	0.128	0.063	0.075	0.028

Table 4.1. Fold change (A) and Statistical significance (B) values for the cell density dependant comparison. Cells were seeded in type I collagen at either 0.5 or 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 0-48 hours of strain at 5% at 1Hz. mRNA of a selection of metalloproteinase and matrix genes were measured using standard qRT-PCR. The Wilcoxon signed rank test was used to test statistical significance, the chosen cut off for significance was p=0.05. Values highlighted in grey were below p=0.05 and therefore significant. P values printed in white font and highlighted in darker grey were <0.01 and therefore significant. (n≥6).

4.3.2. Metalloproteinase family and TIMP family analysis

The expression of 18 ADAMTS genes, all 23 MMP genes and all 4 TIMP genes were analysed in samples treated with or without strain at 24 and 48 hours. Of the 18 ADAMTS genes and 23 MMP genes ADAMTS8, ADAMTS15, ADAMTS18, ADMATS19, MMP12, MMP20, MMP21, MMP25, MMP26 and MMP28 were not detected (Average Ct>37). Genes with low expression (Ct between 35 and 37) included ADAMTS9, ADAMTS16 and ADAMTS17, MMP1, MMP3, MMP7, MMP8, MMP10, MMP15, MMP24 and MMP28.

The following genes showed a significant increase after 24 hours mechanical strain: ADAMTS2 (2.5 fold), ADAMTS4 (3.2 fold), ADAMTS6 (3.6 fold), ADAMTS14 (3 fold), ADAMTS16 (25.6 fold), MMP10 (53.5 fold), MMP24 (5.2 fold) and TIMP3 (4.6 fold) (Fig. 1.2). At 48 hours the following genes were increased; ADAMTS2 (2 fold), ADAMTS4 (7.1 fold), ADAMTS10 (2.6 fold), ADAMTS16 (24.1 fold), MMP24 (35.2 fold) and TIMP3 (5.3 fold). At 48 hours there was a decrease with strain in MMP3 (33.3 fold), MMP10 (4 fold), MMP11 (1.6 fold) and MMP17 (2 fold) (For statistical or fold changes see table 4.2).

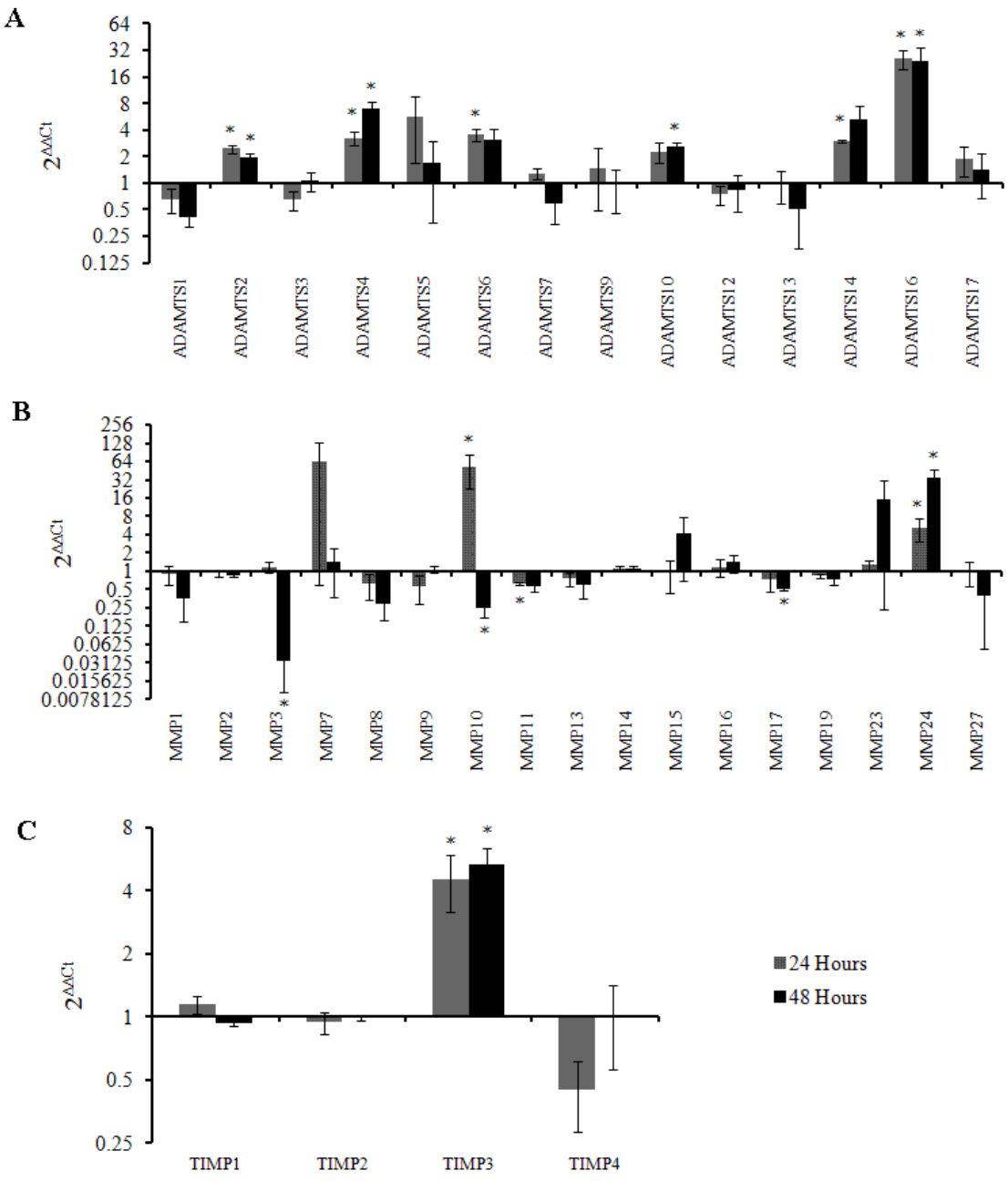

Figure 4.2. Metalloproteinase and TIMP family expression in response to mechanical load: **TLDA analysis at 24 and 48 hours**. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 24 and 48 hours of strain at 5% at 1Hz. mRNA analysis of ADAMTS (A), MMP (B) and TIMP (C) genes was measured using TLDA qRT-PCR. The grey bars represent 24 hour and black bars represent 48 hour time points. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * according to the paired t test (2 way) ($p<0.05$).

	24 hours		48 hours		Average Ct
	Fold change	P value	Fold change	P value	
ADAMTS1	0.66	0.2216	0.41	0.0532	33
ADAMTS2	2.46	0.0114	1.97	0.0254	30
ADAMTS3	0.65	0.2365	1.06	0.9652	34
ADAMTS4	3.24	0.0221	7.10	0.0095	31
ADAMTS5	5.72	0.2049	1.68	0.5939	34
ADAMTS6	3.59	0.0185	3.10	0.1469	32
ADAMTS7	1.30	0.2282	0.58	0.2475	33
ADAMTS9	1.49	0.7651	0.95	0.5596	36
ADAMTS10	2.29	0.1127	2.63	0.0075	31
ADAMTS12	0.74	0.2959	0.85	0.4968	32
ADAMTS13	0.99	0.7241	0.51	0.4454	34
ADAMTS14	2.99	0.0044	5.19	0.0776	33
ADAMTS16	25.63	0.0047	24.10	0.0228	35
ADAMTS17	1.88	0.2765	1.42	0.3994	35
MMP1	0.89	0.4521	0.35	0.1712	36
MMP2	0.95	0.6273	0.84	0.0694	23
MMP3	1.17	0.6612	0.03	0.0390	37
MMP7	64.52	0.9163	1.39	0.5380	35
MMP8	0.62	0.2635	0.29	0.2153	36
MMP9	0.56	0.3063	1.07	0.6702	33
MMP10	53.52	0.0205	0.25	0.0309	37
MMP11	0.63	0.0098	0.57	0.1012	32
MMP13	0.76	0.3234	0.60	0.2668	31
MMP14	1.11	0.3778	1.10	0.5518	25
MMP15	0.99	0.5702	4.16	0.6765	35
MMP16	1.19	0.8899	1.40	0.6636	34
MMP17	0.71	0.2920	0.51	0.0067	32
MMP19	0.83	0.1745	0.73	0.2121	33
MMP23	1.28	0.4269	15.49	0.9921	34
MMP24	5.16	0.0489	35.20	0.0067	36
MMP27	0.99	0.6102	0.39	0.2084	38
TIMP1	1.15	0.3687	0.93	0.1554	24
TIMP2	0.95	0.5946	1.00	0.9283	25
TIMP3	4.55	0.0229	5.31	0.0099	27
TIMP4	0.45	0.2016	0.99	0.6756	34

Table 4.2. Fold change and statistical significance values for Metalloproteinase and TIMP family analysis. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 24 and 48 hours of strain at 5% at 1Hz. mRNA analysis of a selection of metalloproteinase and TIMP genes were measured using TLDA qRT-PCR (n=3). Genes highlighted in grey have an average Ct of >35. Values highlighted in red indicate that there is a significant change and $p<0.05$.

4.3.3. Time course analysis of selected genes

Time course analysis included time points 2, 4, 8, 24 and 48 hours and a select number of metalloproteinase genes selected on the basis of responses seen at 24 and 48 hours in the previous TLDA as well as a range of matrix genes, cytokines and tendon markers,. Of these genes MMP8, ADAMTS16, COL2A1, Tenomodulin, IL1β, IL4, IL17, TNF and metabotropic glutamate receptor (GRM) 5 and GRM6 were not sufficiently detected. MMP3 had low expression across the range of conditions (Ct>35).

There were no significant changes in collagen expression. Of the proteoglycans investigated, Aggrecan was decreased with loading at 4 hours (1.4 fold) (Fig 4.4). Decorin was decreased with strain at 2 hours (1.3 fold). Lumican was decreased significantly with strain at 48 hours (1.4 fold). Versican showed a similar pattern of regulation to decorin and was decreased with strain at 8 hours (1.2 fold, p=0.004), but this decrease was not maintained. Thrombospondin-1 was increased with strain at 24 hours (p=0.045).

ADAMTS genes selected for TLDA analysis were ADAMTS4, ADAMTS5, ADAMTS6 and ADAMTS10 as they showed some of the most robust changes seen in the metalloproteinase and TIMP TLDA in terms of ADAMTS genes. ADAMTS10 was the only ADAMTS to be significantly increased with strain (2.8 fold) at 24 hours (Fig 4.5).

MMP1, MMP2, MMP3, MMP8, MMP10 and MMP13 were selected for analysis of the time course experiments due to interesting responses at 24 and 48 hours in the metalloproteinase and TIMP TLDA. MMP2 was significantly decreased with mechanical load (1.3 fold) at 48 hours (Fig 4.5). MMP10 was not significantly regulated by strain at 48 hours; however there was a significant increase at 2 hours of strain (1.16 fold).

Of the four TIMPs; TIMP1, TIMP2 and TIMP3 were chosen for analysis. TIMP4 was omitted on the grounds that we were unable to consistently measure TIMP4 response to strain using standard qRT-PCR. TIMP1 and TIMP2 were not significantly regulated with mechanical load (Fig 4.5). TIMP3 was significantly increased with strain (5.9 fold) at 48 hours.

Cytokines and cytokine receptors often associated with tendinopathy were selected, these included IL6, IL6R, IGF, COX2, TGFβ and CTGF. IL6R was significantly decreased at 2

(1.28 fold) and 48 (4.2 fold) hours with strain. IGF1 was significantly increased with strain (2.3 fold) at 24 hours. There was no significant change in CTGF, COX2 or TGFβ.

Despite the lack of true 'markers' of tendon phenotype, genes indicative of tendon cell phenotype were selected to identify whether cells had deviated from the tendon phenotype, these were tenomodulin, scleraxis and SOX9. However we were unable to detect tenomodulin in any of our samples. There were no significant changes in Scleraxis or SOX9. Fold changes and statistical p values for all genes in the metalloproteinase and TIMP family TLDA are shown in tables 4.3 and 4.4.

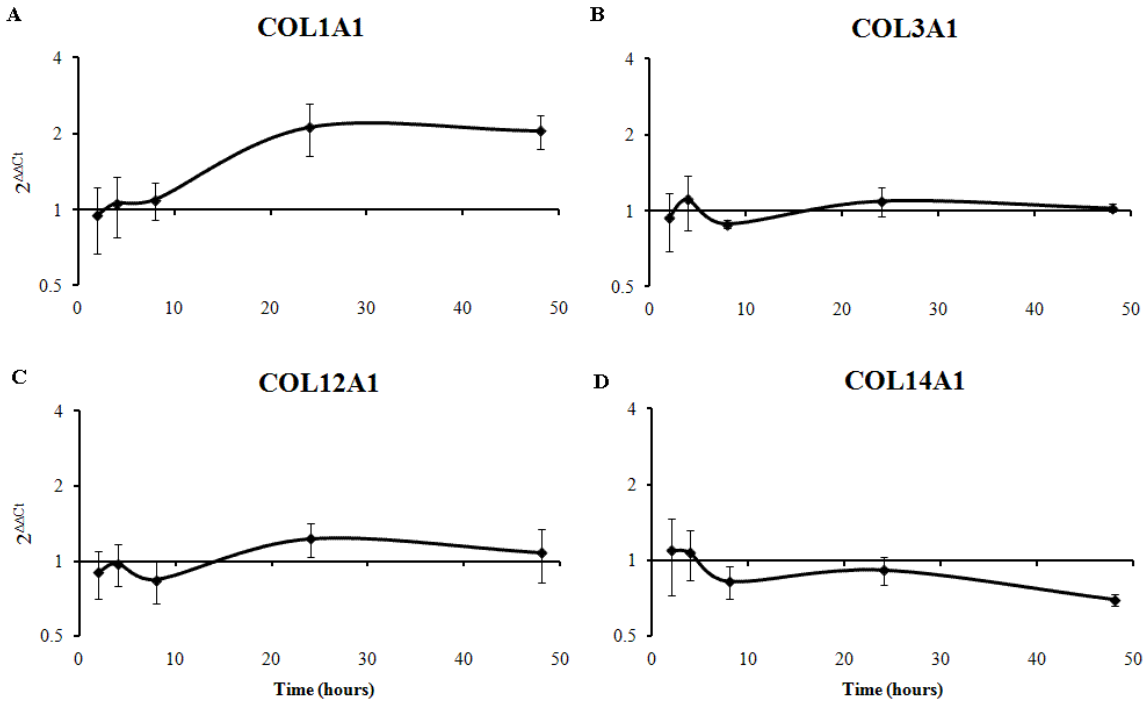

Figure 4.3. Regulation of Collagen expression with mechanical loading: TLDA time course 0-48 hours. TLDA analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. COL1A1 (A), COL3A1 (B), COL12A1 (C) and COL14A1 (D) were analysed using TLDA. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Statistical analysis was performed using the Student's t-test (2 way, paired). Significance is denoted as * (p=0.05).

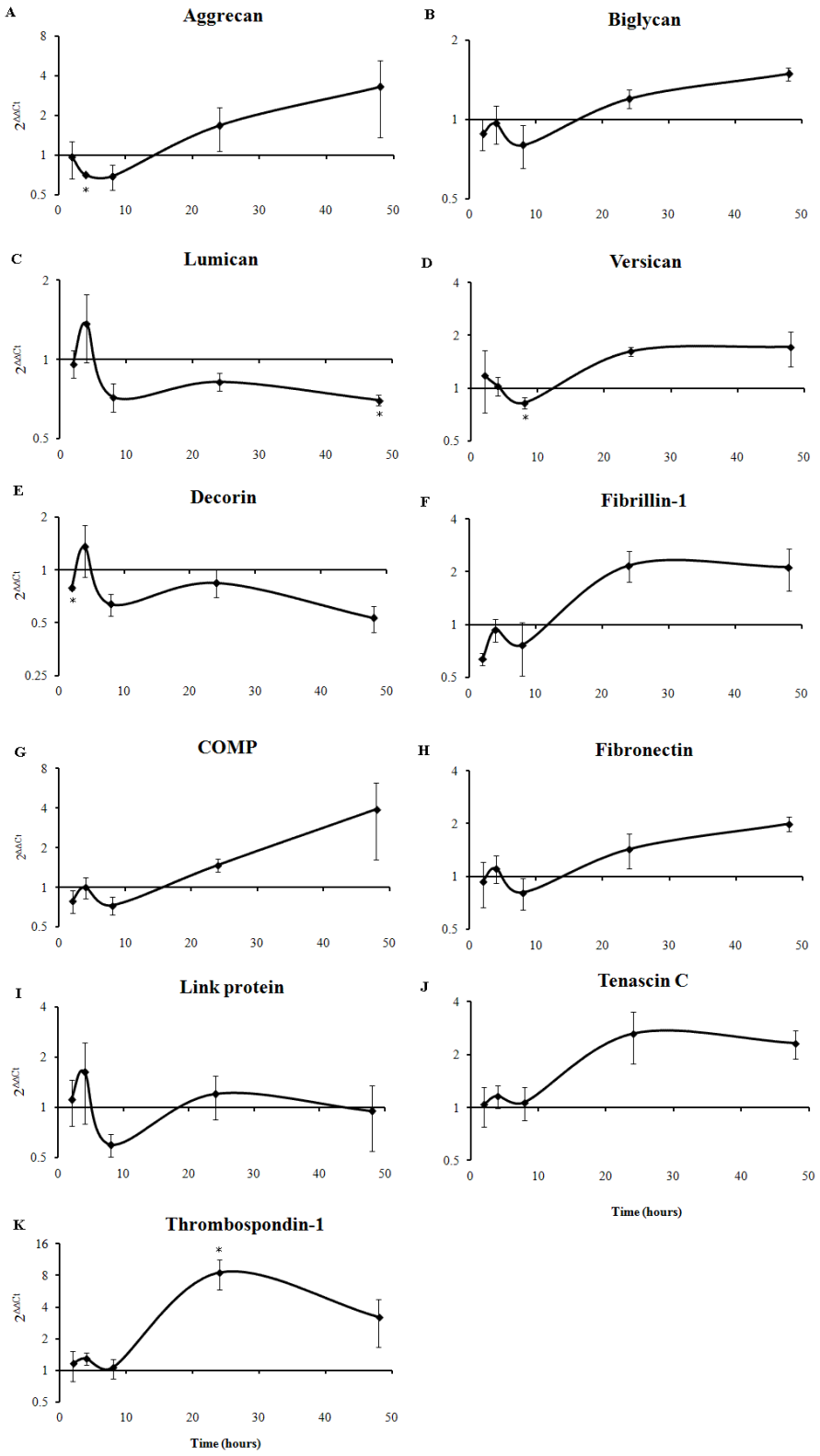

Figure 4.4. Regulation of Matrix gene expression with mechanical loading: Time course TLDA 0-48 hours. TLDA analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Aggrecan (A), Biglycan (B), Lumican (C), Versican (D), Decorin (E), Fibrillin-1 (F), COMP (G), Fibronectin (H), Link protein (I), Tenascin-C (J) and Thrombospondin-1 (K) were measured using TLDA. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Statistical analysis was performed using the Student's t-test (2 way, paired). Significance is denoted as * (p=0.05).

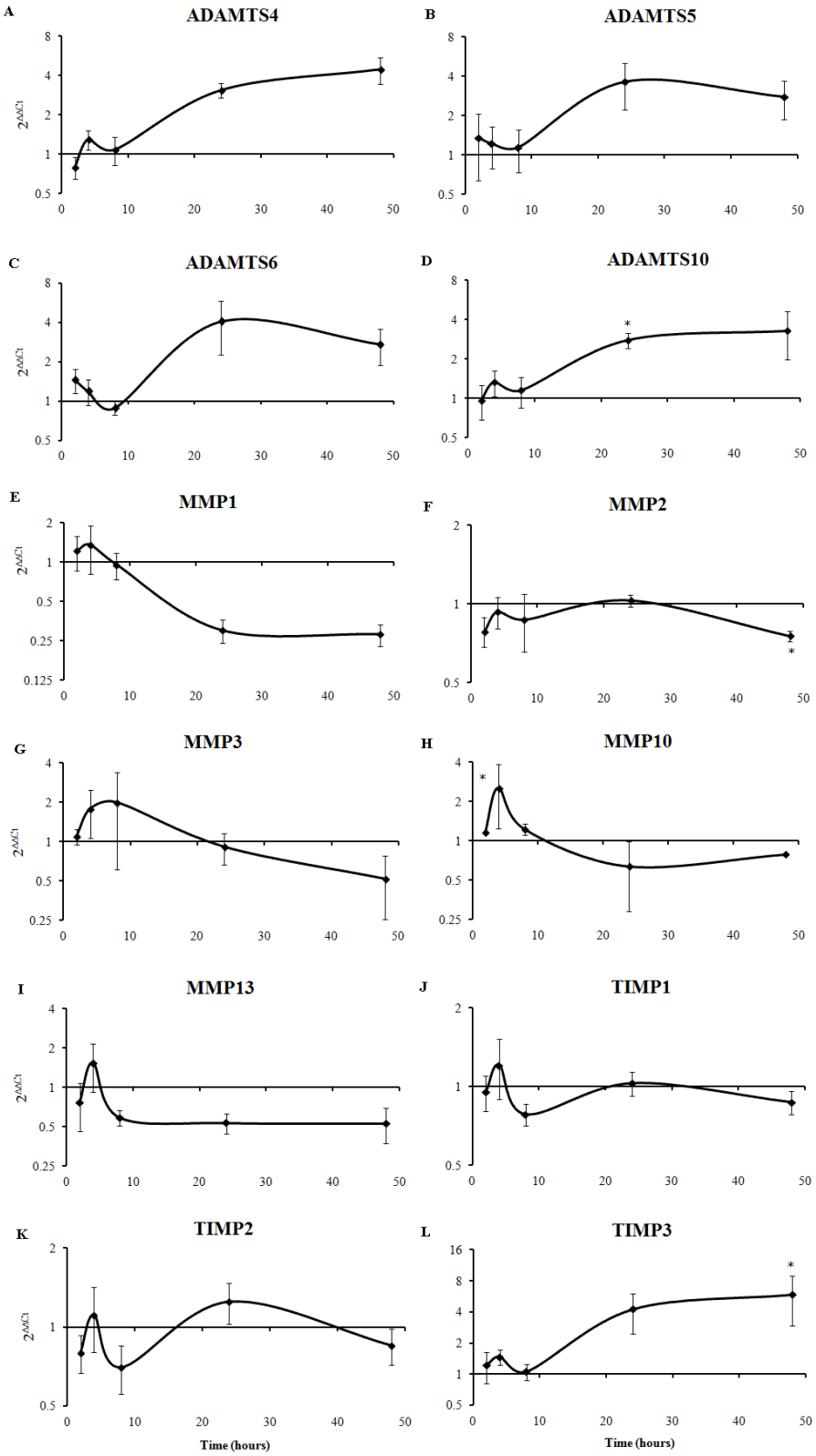

Figure 4.5. Regulation of metalloproteinases with mechanical loading: Time course TLDA 0-48 hours. TLDA analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. ADAMTS4 (A), ADAMTS5 (B), ADAMTS6 (C), ADAMTS10 (D), MMP1 (E), MMP2 (F), MMP3 (G), MMP10 (H) MMP13 (I), TIMP1 (J), TIMP2 (K) and TIMP3 (L) were analysed using TLDA. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Statistical analysis was performed using the Student's t-test (2 way, paired). Significance is denoted as * (p=0.05).

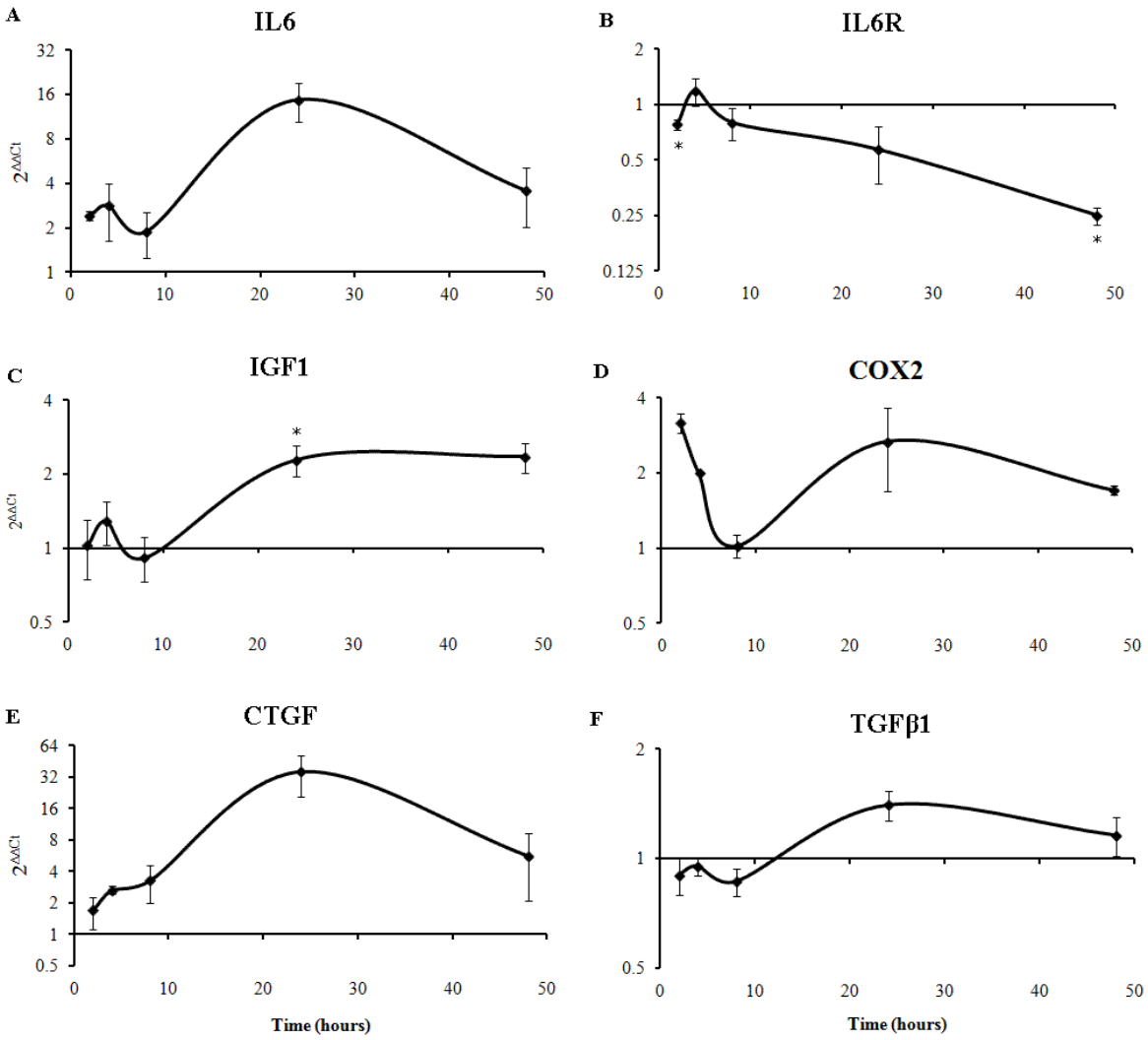

Figure 4.6. Regulation of cytokine expression with mechanical loading: Time course TLDA 0-48 hours. TLDA analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. IL6 (A), IL6R (B), IGF1 (C), COX2 (D), CTGF (E), TGFβ1 (F) were analysed using TLDA. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Statistical analysis was performed using the Student's t-test (2 way, paired). Significance is denoted as * (p=0.05).

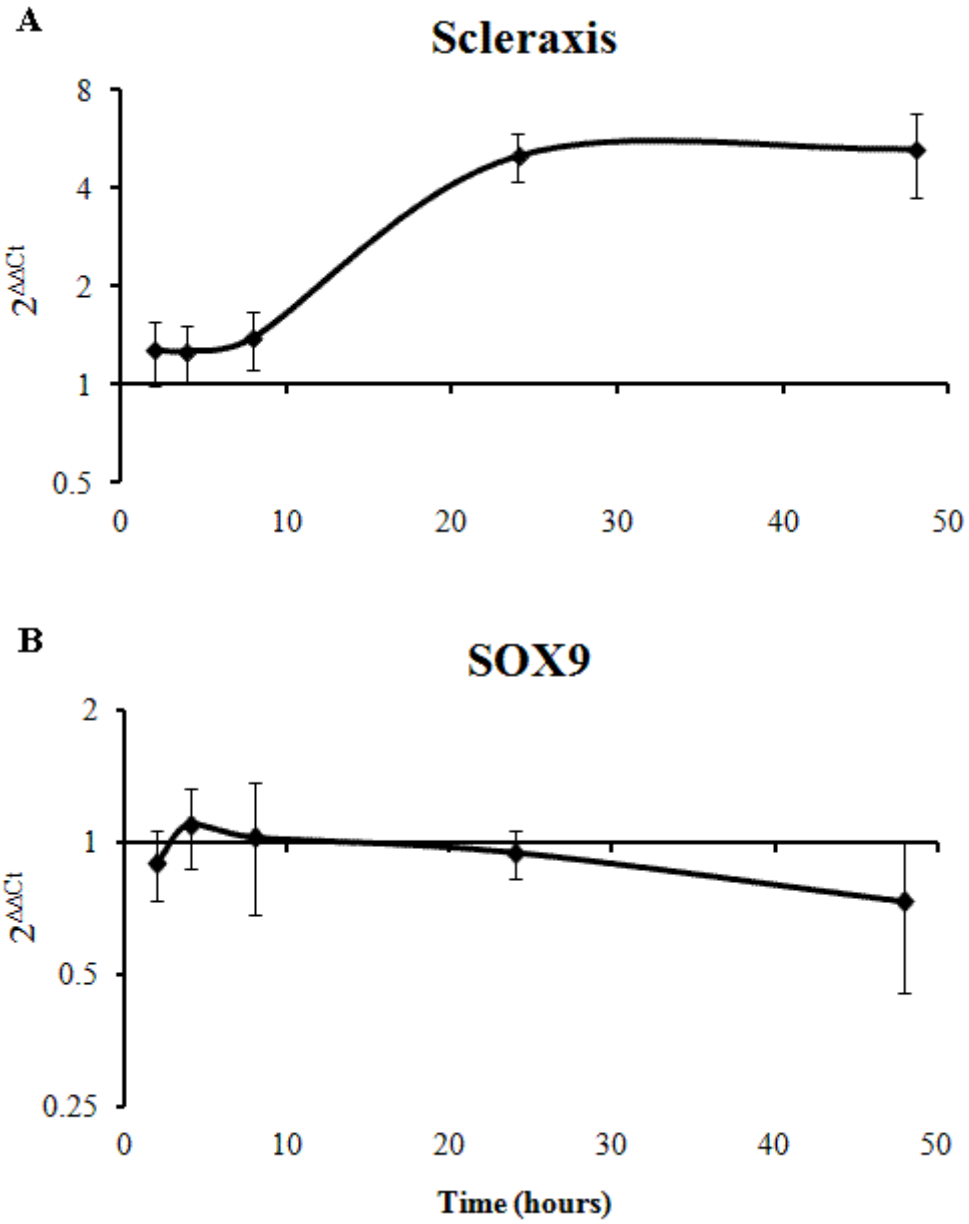

Figure 4.7. Regulation of cell lineage 'markers' with mechanical loading: Time course TLDA 0-48 hours. TLDA analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Scleraxis and SOX9 were measured using TLDA. Data was normalised to 18s and presented as a mean (n=3, ± SE) fold change with strain ($2^{\Delta\Delta Ct}$). Statistical analysis was performed using the Student's t-test (2 way, paired). Significance is denoted as * (p=0.05).

	Time (hours)				
	2	4	8	24	48
ADAMTS4	0.796	1.296	1.086	3.091	4.461
ADAMTS5	1.354	1.222	1.148	3.629	2.780
ADAMTS6	1.459	1.187	0.886	4.060	2.715
ADAMTS10	0.968	1.335	1.153	2.804	3.304
Aggrecan	0.963	0.706	0.690	1.677	3.293
Biglycan	0.881	0.967	0.801	1.198	1.486
COL1A1	0.949	1.059	1.094	2.125	2.053
COL3A1	0.935	1.109	0.886	1.095	1.024
COL12A1	0.904	0.978	0.842	1.233	1.082
COL14A1	1.095	1.072	0.824	0.915	0.697
COMP	0.791	1.007	0.732	1.478	3.934
COX2	3.193	1.995	1.022	2.676	1.707
CTGF	1.700	2.652	3.271	36.274	5.657
Decorin	0.786	1.355	0.638	0.846	0.533
Fibrillin-1	0.640	0.940	0.767	2.175	2.126
Fibronectin	0.938	1.115	0.810	1.433	2.002
IGF1	1.024	1.289	0.915	2.281	2.351
IL6	2.409	2.818	1.887	14.722	3.572
IL6R	0.781	1.180	0.795	0.568	0.250
Link Protein	1.118	1.628	0.597	1.200	0.948
Lumican	0.964	1.371	0.720	0.822	0.700
MMP1	1.227	1.356	0.955	0.302	0.282
MMP2	0.786	0.936	0.873	1.031	0.756
MMP3	1.095	1.775	1.995	0.913	0.519
MMP10	1.160	2.537	1.233	0.640	0.789
MMP13	0.772	1.539	0.590	0.537	0.532
Scleraxis	1.276	1.267	1.390	5.047	5.298
SOX9	0.899	1.097	1.023	0.944	0.729
Tenascin C	1.044	1.165	1.071	2.635	2.324
TGFβ1	0.901	0.952	0.864	1.402	1.158
Thrombospondin 1	1.162	1.296	1.058	8.565	3.210
TIMP1	0.956	1.209	0.784	1.031	0.872
TIMP2	0.798	1.110	0.702	1.248	0.849
TIMP3	1.222	1.470	1.052	4.264	5.910
Versican	1.181	1.035	0.828	1.623	1.721

Table 4.3. Fold change values for Time course for selected gene analysis. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 0-48 hours of strain at 5% at 1Hz. mRNA analysis of a selection of metalloproteinase and matrix genes were measured using TLDA qRT-PCR (n=3).

	Time (hours)					Average C
	2	4	8	24	48	
ADAMTS4	0.382	0.375	0.533	0.128	0.111	28
ADAMTS5	0.718	0.561	0.883	0.088	0.172	33
ADAMTS6	0.311	0.743	0.335	0.174	0.204	29
ADAMTS10	0.702	0.385	0.642	0.047	0.153	29
Aggrecan	0.752	0.047	0.176	0.354	0.274	27
Biglycan	0.290	0.586	0.392	0.250	0.099	21
COL1A1	0.400	0.811	0.473	0.190	0.129	20
COL3A1	0.638	0.968	0.138	0.584	0.517	22
COL12A1	0.322	0.642	0.500	0.405	0.836	24
COL14A1	0.847	0.960	0.291	0.563	0.053	27
COMP	0.369	0.659	0.249	0.058	0.098	26
COX2	0.095		0.929	0.420	0.127	31
CTGF	0.305	0.164	0.333	0.089	0.301	24
Decorin	0.019	0.527	0.175	0.434	0.059	26
Fibrillin-1	0.074	0.681	0.364	0.139	0.234	26
Fibronectin	0.441	0.648	0.364	0.292	0.062	18
IGF1	0.860	0.377	0.719	0.041	0.228	27
IL6	0.055	0.215	0.208	0.169	0.346	31
IL6R	0.020	0.801	0.256	0.092	0.036	31
Link Protein	0.517	0.830	0.273	0.623	0.566	31
Lumican	0.933	0.623	0.117	0.188	0.021	25
MMP1	0.712	0.580	0.811	0.120	0.637	32
MMP2	0.273	0.726	0.381	0.585	0.031	20
MMP3	0.894	0.842	0.666	0.328	0.399	36
MMP10	0.023	0.507	0.235	0.382		35
MMP13	0.227	0.599	0.304	0.357	0.372	29
Scleraxis	0.573	0.632	0.190	0.167	0.115	25
SOX9	0.498	0.960	0.821	0.605	0.337	31
Tenascin C	0.556	0.615	0.594	0.242	0.236	23
TGFβ1	0.446	0.468	0.287	0.237	0.524	23
Thrombospondin 1	0.812	0.070	0.388	0.045	0.295	23
TIMP1	0.853	0.449	0.311	0.695	0.230	20
TIMP2	0.269	0.956	0.193	0.356	0.421	21
TIMP3	0.884	0.290	0.696	0.096	0.039	25
Versican	0.649	0.960	0.004	0.055	0.214	28

Table 4.4. Statistical significance values for Time course of selected genes TLDA. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 0- 48 hours of strain at 5% at 1Hz. mRNA analysis of a selection of metalloproteinase and matrix genes were measured using TLDA qRT-PCR (n=3). Genes highlighted in grey have an average Ct of >35. Values highlighted in red indicate that there is a significant change and $p<0.05$.

4.3.4. Comparison of normal and pathological tenocytes

All metalloproteinase and matrix gene responses we have mentioned above are those observed in cells collected from patients with Achilles tendinopathy, however, we are also interested in whether the same responses were seen in cells derived from patients with ruptured tendon or normal tendon phenotype. Single cell isolates derived from each of these cell lineages were compared in terms of MMP1 and MMP13 expression (Fig 4.8). MMP1 (14.3, 2.2 and 6.5 fold) and MMP13 (6.3, 3, 2 fold) were decreased with mechanical loading in all cell lineages (normal, tendinopathy and ruptured respectively) despite the differences in disease phenotype.(Figure 4.8).

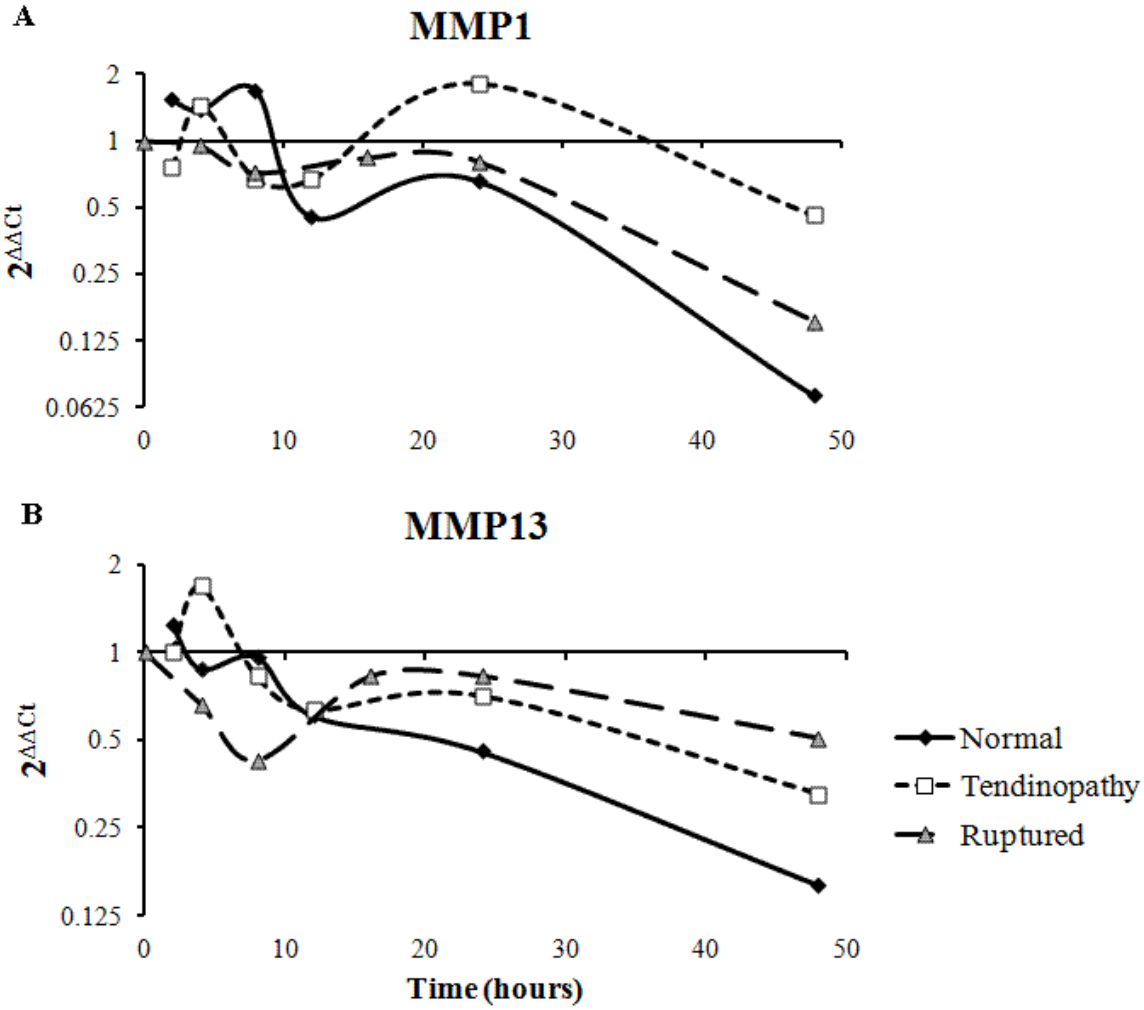

Figure 4.8. MMP1 (A) and MMP13 (B) regulation with mechanical loading in cells derived from normal, tendinopathic and ruptured tendon. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 0-48 hours of strain at 5% at 1Hz. mRNA of MMP1 and MMP13 was measured using standard qRT-PCR. Data was normalised to TOP1 and presented as a fold change with strain ($2^{\Delta\Delta Ct}$) (n=1).

4.3.5. MMP2 protein expression

Unprocessed (72kDa) and processed (68kDa) forms of MMP2 were detectable at all time points (Figure 4.9). These correspond to inactive (proMMP2, 72kDa) and active forms of MMP2 (MMP2, 68kDa) respectively, however this assay does not confirm MMP2 activity of the sample outside the assay. MMP2 is increased in both processed and unprocessed forms during the time course; this may be due to accumulation of MMP2 in the medium during the time course as the medium was not replaced during this period. There is no difference between the level of either processed or unprocessed MMP2 between strain and non-strain cultures at 2, 4, 24 or 48 hours, however at 8 hours of strain there was an increase in the level of processed MMP2 compared to non-strained controls (1.54 fold, p=0.0219). We were unable to sufficiently detect either unprocessed or processed MMP9 protein using gelatin zymography (data not shown).

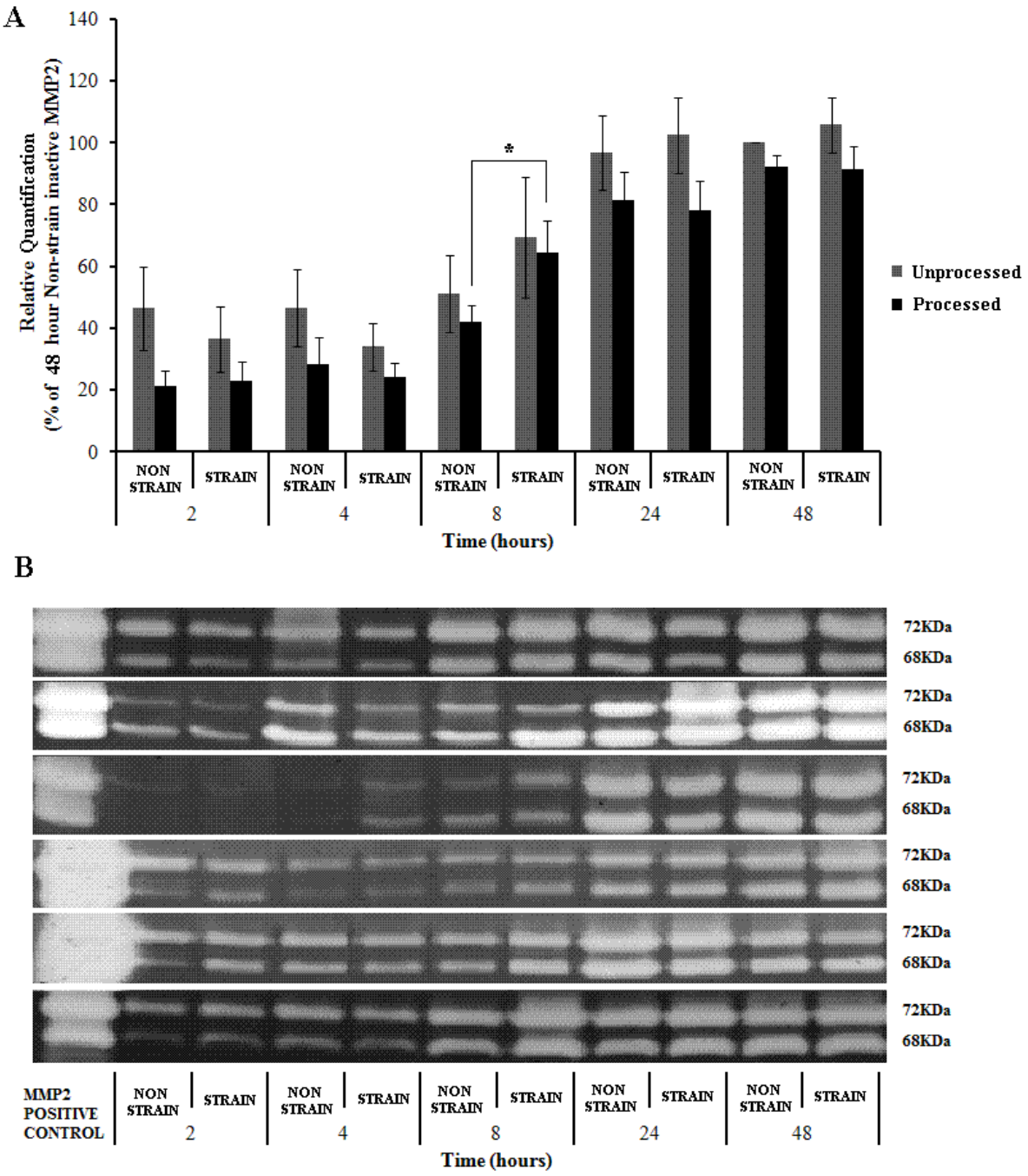

Figure 4.9. Gelatin zymogram of MMP2 protein expression in strain conditioned media. Cells were seeded in type I collagen at 1.5 x 10^6 cells/ml into Flexcell tissue train plates and subjected to 0-48 hours of strain at 5% at 1Hz. Conditioned media was analysed using gelatin zymography. Zymograms were imaged and bands were quantitated using image j software. Data was expressed as a percentage of 2 hour non-strained unprocessed MMP2 levels, the mean (± SE) of 6 separate experiments (A). Zymograms from 6 different cell isolates are shown (B). The 68kDa band corresponds to the unprocessed form of MMP2, The 72kDa band corresponds to the processed form of MMP2. Statistical analysis was performed using the t test (2 way, paired t test). Significance is denoted as * (n=6) (p=0.05).

4.4. DISCUSSION

We have shown that mRNA expression of MMP1, MMP2, MMP3, MMP13 and fibulin-1 are significantly decreased after 48 hours and ADAM12 and matrix genes COL1A1 and Elastin are significantly increased in response to 24 hours of 0-5% cyclic loading at 1Hz in tendinopathy derived tenocytes. Our results show that gene responses were more robust in cells seeded at the higher cell density therefore cell interactions may be involved in the mechanoregulation of these genes. However, more detailed study is required to confirm this. Using TLDA analysis of a wide range of metalloproteinase, TIMPs, cytokines, cell lineage markers and collagens, we can see that there is an overall increase in ADAMTS, tendon cell lineage markers and collagen expression, and a decrease in MMP expression at 24 and 48 hours. There was therefore a general anabolic response to mechanical load. Our data indicates that not only do tendinopathy derived cells respond to mechanical loading but cells derived from normal and ruptured tendon also show a similar pattern of regulation, with normal tendon derived cells showing a more marked pattern of response in preliminary experiments. It is possible that cells derived from ruptured and tendinopathic tissues are less responsive to mechanical load. However, due to time constraints and relative paucity of normal tendon we were only able to repeat this experiment in one cell isolate. To confirm this more cell isolate repeats are required.

Data from the TLDA experiments could not be combined with standard qRT-PCR analysis because the target amplicons of primers and probe sets were different on the TLDA compared to these used for standard qRT-PCR. The consistency in the majority of gene changes between standard qRT-PCR analysis and TLDA analysis with different primer and probe sets increases confidence in gene changes

Some genes were analysed both by TLDA and the standard qRT-PCR analysis. The majority of gene changes were consistent between TLDA and standard qRT-PCR analysis, however a number of changes were significant in the standard qRT-PCR analysis and not in TLDA analysis, for example there were significant changes in MMP1, MMP3 and MMP13 with mechanical load using standard qRT-PCR, however despite a trend to decrease these genes were not significantly regulated with mechanical load in the TLDA. The lack of statistical significance in the TLDA experiments compared to standard qRT-PCR could be explained by the fact that TLDA experiments were not sufficiently powered:

they were only repeated 3 times compared to an n≤6 in standard qRT-PCR experiments. Also TLDA analysis utilises a lower level of RNA and would therefore yield more variable results in genes of low expression. MMP10 however was shown to increase with strain at 24 hours the preliminary metalloproteinase and matrix gene TLDA, whereas there was no change in MMP10 at 24 hours in the time course TLDA. This may be due to variation in response according to the cell isolates studied.

In the current study genes were chosen for analysis on the basis that they are reportedly modified by mechanical load or tendinopathy in some shape or form. However a number of genes we chose for analysis were not detected. The glutamate receptors, metabotropic glutamate receptor (GRM) 5 and GRM6 were measured as previous studies have shown an increase in glutamate in patellar tendinopathy and its association with pain (Alfredson, Thorsen et al. 1999; Alfredson, Forsgren et al. 2001), however neither GRM5 nor GRM6 were sufficiently detected. This indicates that glutamate receptors are not expressed in passaged tenocytes, however because we did not include a positive control we cannot confirm this. IL4 and IL17 were also not detected. We chose to measure IL17 as it was decreased at the mRNA level in diseased tendon in a human study focusing on patients with chronic tendinopathy (Jelinsky, Rodeo et al. 2011). IL4 is implicated in the strain mediated regulation of MMP3 and ADAMTS4 mRNA (but not aggrecan or collagen) in invertebral disc annulus fibrosus cells (Gilbert, Hoyland et al. 2011). Therefore we measured IL4 mRNA to assess the cytokines role in tenocyte mechanotransduction. Both IL4 and IL17 are inflammatory cytokines, therefore we wanted to measure their response to mechanical loading as it remains uncertain what role inflammation plays in the development of tendinopathy. Due to the fact that neither IL4 nor IL17 were detectable in our study, it is therefore likely that cultured tenocytes do not express either of these proteins and that IL4 is not involved in tenocyte mechanotransduction (at least in vitro). TNF is an inflammatory cytokine involved in the regulation of cellular apoptosis. Apoptosis is reported to increase in athletes with patellar tendinopathy (Lian, Scott et al. 2007). TNF has been shown to increase in rotator cuff disease (Voloshin, Gelinas et al. 2005) and also increase with stress deprivation in the patellar tendon (Uchida, Tohyama et al. 2005) and in rat tail tendon studies (Egerbacher, Arnoczky et al. 2008). Therefore we chose to measure TNF mRNA. However in our TLDA, TNF gene expression was undetectable. This indicates that TNF is not expressed in cultured tenocytes.

We are the first to study the effect of mechanical regulation on an array of protease genes in human tenocytes. We have chosen 5% uniaxial strain as we believe cells undergo similar levels of strain in vivo. Although it is extremely difficult to determine what levels of strain are physiologically relevant, our data shows that 5% mechanical strain has a potential anabolic effect on the collagenous matrix, as three collagenases (MMP1, MMP8 and MMP13) are decreased at the mRNA level with mechanical strain, accompanied by an increase in COL1A1 expression. Comparing this data to other studies indicates that in the current study strain levels are more physiologically relevant than others using higher strain levels have shown a more catabolic response; i.e. an increase in MMP13 (Asundi and Rempel 2008; Sun, Li et al. 2008).

The responses to strain are more robust in cultures seeded at a high cell density. This may be due to increases in the number of cells resulting in increased cellular proximity and therefore increased the level of cell-cell signalling. These data suggest that an increase in cell-cell communication may play a role in mechanoregulation of metalloproteinase and matrix protein gene expression. However to determine which molecules are involved more specific inhibitory experiments are required, i.e. inhibition of specific integrins and connexins. An early response seen in cultures at the lower cell density only, could be representative of a mechanism of load perception that has less requirement for cell to cell contact. This early response involved an increase in MMP2 and MMP13 as well as a trend to increase in ADAM12 and Elastin. This increase in MMP expression indicates that lack of cell contact may result in a modification in the response to mechanical loading; therefore areas of very low or high cellularity may be affected by strain differently. Differences in cellularity are evident in diseased tendon (Khan, Cook et al. 1999), therefore changes in cellular communication may play a key role in tendinopathy.

We have described an overall anabolic response in metalloproteinase and matrix gene expression which indicates that there is a reduction of matrix turnover. Potential anti-cancer drugs were trailed in the 1990's that targeted metalloproteinase activity, these included doxycycline and illomastat (GM6001) (Drummond, Beckett et al. 1999; Tierney, Griffin et al. 1999). It became clear that these anti-cancer drugs induced side effects that resembled tendinopathies; therefore a certain level of metalloproteinase activity is required for normal tendon homeostasis. This suggests that the reduction in matrix turnover in response to strain may not be a positive response. In theory, the reduction in turnover in response to high levels of strain may induce a weakening of the tendon and potential

tendinopathic phenotype after prolonged exposure. However, despite the decrease in metalloproteinase mRNA, metalloproteinases are still expressed in strained tenocytes in the current study; therefore turnover can still occur. However, it still remains to be seen whether the level of turnover is sufficient to maintain a healthy balance.

We have also measured the levels of proMMP2 and MMP2 protein expression in response to mechanical load; we showed a 1.5 fold increase in active MMP2 after 8 hours of strain compared to non-strained controls. Both proMMP2 and MMP2 were increased over the time course peaking at 48 hours and at 48 hours there was no difference between strained and non-strained cultures in terms of MMP2. This indicates that despite the reduction in MMP2 mRNA expression we still see increased levels of gelatinase activity and therefore matrix turnover may be maintained. The mechanism of strain induced activation of MMP2 at 8 hours is unknown, however MMP24 which is stimulated at 24 and 48 hours with strain in the current study, may be involved as it plays a role in the activation of proMMP2 (Visse and Nagase 2003). To confirm this mRNA and protein analysis of MMP24 would be required at a time point that precedes the activation in MMP2 in response to load. A limitation of this study is the lack of protein data. We were unable to measure the protein expression of other MMPs due to the low levels of detection in both strained and non-strained samples without cytokine stimulation (using western blotting techniques). Measurement of collagen degradation (using the Hydroxyproline assay or running protein extracts on a SDS-PAGE gel to assess collagen breakdown), metalloproteinase protein (using specific ELISAs) and activity of metalloproteinases (specific fluorescent substrates) would be the next step in understanding the level of turnover that is occurring in response to load.

There have been a number of studies that have looked at the effects of mechanical strain on MMPs, mainly in rodent tendon in vivo, ex vivo or in vitro cell studies. Obvious limitations of these studies include the differences in species as rodents do not express MMP1; instead they only express MMP13, which is the nearest homologue. In some studies 2D models lack the cell-matrix contact that is so obviously important in the 3D matrix seen in vivo. Moderate levels of strain are considered to be <6% (Lavagnino, Arnoczky et al. 2003; Screen, Lee et al. 2004), Studies focusing on a similar regime are reported to have an anabolic effect upon the tendon, consistent with the current study. For example in rabbit flexor tendon studies MMP3 was decreased with ≤4MPa (Asundi and Rempel 2008) and rat tail tendon studies ex vivo and in vitro have revealed a decrease in

MMP13 following 1% cyclic strain (Arnoczky, Lavagnino et al. 2008) or 3% cyclic strain superimposed on a static strain of 2% (Maeda, Fleischmann et al. 2010). Similarly static or cyclic loading at loads of up to 2.6MPa (Arnoczky, Tian et al. 2004) or 6% cyclic load (up to 1Hz) (Lavagnino, Arnoczky et al. 2003) have been reported to down-regulate MMP13 (MMP1 homologue) mRNA in rat tail tendon fascicles after 24 hours. Sun and colleagues have cyclically loaded (1Hz) rat patella tendons in vivo and cells in vitro, and in both instances observed a decrease in MMP13 (mRNA and protein) with load; cells at <5MPa or tendon until 0.6% elongation (Sun, Li et al. 2008). However, a number of rodent tendon in vivo and in vitro studies have shown no significant change in MMP3 or MMP13 mRNA upon moderate loading (Archambault, Tsuzaki et al. 2002; Asundi and Rempel 2008; Asundi and Rempel 2008; Maeda, Fleischmann et al. 2010), this may be due to a lower frequency in strain (<0.5Hz) or the reduced loading time. This is consistent with our data as MMP1, MMP3 and MMP13 are not significantly regulated by strain until 48 hours.

At higher strain levels (more than 1.7% tendon elongation or 4-7.5MPa in the rat) in both in vivo and in vitro tendon MMP13 mRNA was significantly increased (Asundi and Rempel 2008; Sun, Li et al. 2008). Koskinen and colleagues have shown a decrease in proMMP2 protein immediately after exercise and an increase after 3 days in human runners peritendinous tissue dialysate (Koskinen, Heinemeier et al. 2004). In addition a rat in vivo study showed an increase in MMP2 mRNA with different strain regimes via sciatic nerve stimulation (Heinemeier, Olesen et al. 2007). This data is not entirely in agreement with our studies in terms of MMP2 and MMP13 mRNA regulation with strain, although we do show an increase in active MMP2 protein after 8 hours of strain. Again these differences could be due to the higher stress regimes; this indicates that high levels of strain have a more catabolic effect.

Interestingly Maeda et al showed an initial increase in MMP3 and MMP13 mRNA (after 10 minutes) followed by a progressive decrease until 24 hours in a 3% cyclic strain regime superimposed on a static strain of 2% in rat tendon fascicles (Maeda, Shelton et al. 2009). Our data shows a trend to increase in MMP3 at 4 hours which progressively decreases until 48 hours (however this was not the case with MMP13). Maeda and colleagues showed a decrease in MMP9 and MMP14 after 24 hours of mechanical load (Maeda, Fleischmann et al. 2010), the current study showed a small decrease in MMP9 but no change in MMP14 after 24 hours strain. These differences could be due to the difference in species (rat tendon) and the different strain regime (3% strain superimposed on a 2% static strain).

Strain regulation of other MMPs in tendon is not well published. In our TLDA screen regulation of MMP10 and MMP24 with mechanical strain was highlighted, which has not previously been reported in tendon.

An ovine tendon model reported decreased ADAMTS4 mRNA in overstrained areas of infraspinatus tendon (due to partial width laceration) (Smith, Sakurai et al. 2008). We report that ADAMTS4 mRNA is increased with strain. The difference in regulation may well be due to the less damaging loading applied in this study. ADAMTS1 and ADAMTS5 were reported to decrease after 24 hours of 3% cyclic strain superimposed on a static 2% load in a study on rat tail tendon fascicles (Maeda, Fleischmann et al. 2010). Our array data shows agreement in terms of ADAMTS1 expression; however ADAMTS5 shows the opposite effect. This may be due to the underlying static loads or may relate to the difference in species.

TIMPs 1 and 2 have been shown to increase with mechanical strain in rat tendon at the protein and mRNA level, with TIMP1 mRNA regulation reaching a 6 fold increase (Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007). We have not observed changes in either TIMP1 or TIMP2, but showed an increase in TIMP3 with strain in the current study, again this could be due to differences in the loading regime. TIMP3 is reported to inhibit the activity of ADAM12 (Loechel, Fox et al. 2000), therefore the induction of TIMP3 may be enough to abrogate the activity of ADAM12 despite the fact that ADAM12 is also induced with mechanical loading. TIMP3 has also been reported to cause inhibition of MMP1, MMP2, MMP3, ADAMTS4 and ADAMTS5 at the protein level (Kashiwagi, Tortorella et al. 2001; Visse and Nagase 2003). This indicates that strain has a potential protective effect on not only collagen but also proteoglycan due the inhibitory effects of TIMP3 upon ADAMTS4 and ADAMTS5 (Kashiwagi, Tortorella et al. 2001), at the protein level. As ADAMTS4 and ADAMTS5 are the primary aggrecan degrading enzymes (Gendron, Kashiwagi et al. 2007), this may involve a reduction in the level of aggrecan degradation. ADAMTS1 and ADAMTS15 also possess aggrecanase activity (Porter, Clark et al. 2005), and as ADAMTS15 mRNA was undetected in our cells and ADAMTS1 mRNA was decreased in response to loading we can assume that mechanical loading may reduce the level of aggrecan degradation, indicative of adaption to compressive loading. ADAMTS2 and ADAMTS14 are involved in collagen processing (Colige, Beschin et al. 1995; Colige, Li et al. 1997; Fernandes, Hirohata et al. 2001; Wang, Lee et al. 2003), as COL1A1, ADAMTS2 and ADAMTS14 are increased at the mRNA

level in response to load this indicates that collagen production and processing may occur in response to load. However to confirm this more detailed protein analysis is required.

Mechanical strain has also been shown to increase collagen type I and III protein and overall collagen content in vivo, ex vivo and in vitro tendon studies (mainly in the rat) (Yang, Crawford et al. 2004; Screen, Shelton et al. 2005; Heinemeier, Olesen et al. 2007; Maeda, Shelton et al. 2009; Szczodry, Zhang et al. 2009), the current study shows regulation of collagen type I but little regulation in terms of collagen III, another rat study showed agreement with our data as they showed that there was no change in collagen type III with mechanical load (Legerlotz, Schjerling et al. 2007). Proteoglycan regulation has also been reported in a rat tail tendon model. After a cyclic loading regime of 3% overlaying a 2% static strain for 24 hours decorin, fibronectin and biglycan mRNA was decreased, increased and unchanged respectively (Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010). We have shown a similar pattern of regulation (although not significant) in decorin and fibronectin. However we showed a small trend to increase in biglycan at 48 hours, although this regulation is not significant, it is opposite to changes reported by Maeda. Again this is likely to be due to differences in species and loading regime.

Decorin is the most abundant proteoglycan in tendon (Vogel and Heinegard 1985), however with load mRNA expression is decreased. In addition aggrecan, which is expressed throughout the tendon (Samiric, Ilic et al. 2004), although most predominantly in compressed regions of tendon (Vogel and Koob 1989), shows a trend to increase at the mRNA level. This potentially indicates compressive forces are present as well as tensional forces although aggrecan is also stimulated by growth factors TGFβ (Robbins, Evanko et al. 1997). Despite the increase in COL1A1 mRNA expression, decorin and COL3A1 mRNA are decreased and unchanged respectively in response to mechanical load. Decorin and COL1A1 protein products are involved in type I collagen deposition (Thieszen and Rosenquist 1995; Hakkinen, Strassburger et al. 2000). This indicates that collagen deposition may not occur efficiently despite the increase in collagen type I mRNA expression, to confirm this levels of decorin and collagen should be quantitated at the protein level.

Relatively few studies have looked at cytokine regulation in response to mechanical strain. Rat tendon study of a 3% cyclic strain overlaying a 2% static strain showed an significant

increase in COX2 after 24 hours, they also looked at IL6 and IGF1 however these genes were not significantly regulated (Maeda, Fleischmann et al. 2010). IL6 protein was increased in humans after prolonged running exercise (36km) (Langberg, Olesen et al. 2002). In addition a bovine study of fatigue cyclic loading (30% elongation at 1Hz) reported an increase in IL6 mRNA and decrease in IL6R mRNA (IL6R response in GAPDH normalised data only)(Legerlotz, Jones et al. 2011), no other published studies have looked at IL6R expression in response to mechanical loading. Studies of rabbit and human patella fibroblast also showed an increase in COX2 (2 fold) with 20h 6MPa static strain (Asundi and Rempel 2008) or after 4 hours of 4% cyclic strain at 0.5Hz (Yang, Im et al. 2005) respectively. Immunohistochemical analysis showed that IGF1 protein was markedly increased with mechanical loading after twelve weeks of treadmill exercise in a rat model (Scott, Cook et al. 2007). The current study shows a trend to increase in COX2 and IL6 and a significant increase in IGF1 after 24 hours of cyclic loading analogous to many of the changes described above. Lack of gene responses (IL6 and IGF1) in some studies could be due to differences in species or load regime.

TGFβ mRNA and protein expression is increased in response to mechanical loading in a number of studies in both rat tendon and human patellar fibroblasts (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004; Heinemeier, Olesen et al. 2007; Maeda, Shelton et al. 2009; Maeda, Fleischmann et al. 2010), however in a rat wheel running study they did not show any change in expression (Legerlotz, Schjerling et al. 2007). In the current study we do not show a significant increase in TGFβ mRNA, however there is a very small trend to increase after 24 hours cyclic strain. We have shown an increase in CTGF of 36.27 fold after 24 hours of cyclic loading, although this was not significant. A number of rat studies have looked at the effects of mechanical loading on the expression of CTGF; however none of these studies have shown any significant change (Heinemeier, Olesen et al. 2007; Legerlotz, Schjerling et al. 2007; Maeda, Fleischmann et al. 2010). This difference in response may be due to differences in species and differences in load regime. In fibroblasts CTGF is thought to act as a downstream mediator of TGFβ (Grotendorst 1997); therefore TGFβ may induce the increase in CTGF seen in response to mechanical load. However, this has not been confirmed in the current study.

Rodent tissue studies have also measured IL1β in response to mechanical load, a rat in vivo model showed that a 1.7% cyclic load caused an increase in IL1β, however a 0.6% cyclic load decreased IL1β (Sun, Li et al. 2008), in rabbit flexor tendon tissue strained at 4MPa

for 20 hours IL1 β was decreased 2 fold (Asundi and Rempel 2008). IL1 stimulates MMP expression (Nagase and Woessner 1999) and as MMP expression is decreased in response to load we can only assume that IL1 is not increased and may be decreased in response to load. As we were unable to detect IL1β using the TLDA we cannot confirm that IL1β was regulated with 5% cyclic strain, however we cannot confirm that IL1β is not expressed in our cultures. This may have been due to defective primers and probe, further standard qRT-PCR with validated primers and probes is required.

Few studies have measured the regulation of genes indicative of tendon phenotype in response to mechanical load. Scleraxis was increased with both cyclic and static loading regimes in multipotent stem cells (Scott, Danielson et al. 2011), this indicates that with mechanical load stem cells may become more tendon like. We have also shown a trend to increase in scleraxis expression in response to mechanical loading. Although we do not have true markers of tendon phenotype, this indicates that mechanical load may maintain the tendon phenotype. Tenascin C, another 'marker' of tendon, was increased in response to a compressive loading (Martin, Mehr et al. 2003), here we observe a trend to increase in tenascin C expression after 24 hours of cyclic loading. This indicates that cells may undergo compressive loading as well as tensile strain, as tenascin-C is thought to be up-regulated with compressive load (Martin, Mehr et al. 2003).

No other published studies have looked into the mechanical regulation of the ADAMs, TIMPs 3 and 4 and such a large array of ADAMTS and matrix proteins at either the mRNA or protein level in human tendon. Our data suggests that generally there is a decrease in MMPs (MMP1, -2, -3, -8, -10, -11, -13 and -17) and an increase in collagen at the mRNA level. We have also shown an increase in TIMP3 mRNA which is known to inhibit MMP2 activity (Kashiwagi, Tortorella et al. 2001). An increase in MMP2 after 8 hours in the current study is therefore likely to be inhibited by TIMP3. Although to confirm this TIMP3 protein quantification and activity (achievable by reverse zymography) measurement is required. This supports earlier reports that moderate cyclic strain is largely anabolic, i.e. maintaining the collagen components of the ECM.

Arnoczky's group has shown that mechanical strain is important in tendon ECM homeostasis in that MMPs are increased with the absence of tension (Lavagnino, Arnoczky et al. 2003; Arnoczky, Tian et al. 2004; Arnoczky, Lavagnino et al. 2008; Gardner, Arnoczky et al. 2008), data from Smith et al also support this (Smith, Sakurai et al. 2008).

In addition Arnoczky's group have proposed that as stress deprivation caused by isolated tendon fibre damage due to fatigue loading, may be the key factor in causing tendinopathy as opposed to the overall increased mechanical load of the tendon. They argue that load induced tendon catabolism requires a level of strain that does not occur in the tendon in vivo (Arnoczky, Lavagnino et al. 2007). In the current study we have shown little evidence of load inducing catabolic effects, hence our data support Arnoczky's ideas. Therefore it is important for us to fully elucidate the process of mechanotransduction to truly understand the underlying factors contributing to tendinopathy development.

Comparing the responses of metalloproteinase and matrix genes to strain and changes in human tendinopathy may shed light on the relationship between mechanical loading and tendinopathy development. MMP3 and MMP2 expression shows a significant decrease in ruptured and dysfunctional tendon at the mRNA and protein level (Ireland, Harrall et al. 2001; Riley, Curry et al. 2002; Alfredson, Lorentzon et al. 2003; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006). This correlates with the decreases we have observed in MMP2 and MMP3 regulation with mechanical strain in this study. However, in chronic tendinopathy and dysfunctional posterior tibialis tendon MMP2 was increased, which differs to our response seen with strain (Alfredson, Lorentzon et al. 2003; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012). MMP8 expression was unchanged in tendinopathy (Jones, Corps et al. 2006) whereas we showed a trend to decrease with strain. In addition MMP1 (tendinosis or ruptured tendon), MMP13 (acute tendinopathy, dysfunctional or ruptured tendon) and MMP10 (painful tendinopathy) have been reported to increase, increase and decrease respectively (Fu, Chan et al. 2002; Riley, Curry et al. 2002; Lo, Marchuk et al. 2004; Jones, Corps et al. 2006; Clegg, Strassburg et al. 2007; Corps, Robinson et al. 2012). These changes are opposite to that seen in our study, as we see a decrease in MMP1 and MMP13. In another study MMP1 and MMP13 mRNA were undetectable in tendinopathic tissue (Jelinsky, Rodeo et al. 2011). These similarities and discrepancies in terms of RNA responses in tendinopathic phenotype and strain regulation may indicate that tendon disease may be a result of a complex set of causative factors as well as increased loading.

ADAMTS4 and ADAM12 have been reported to increase in ruptured tendon (Jones, Corps et al. 2006; Corps, Jones et al. 2008; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012), An increase in ADAMTS5 is also reported in dysfunctional posterior tibialis tendon (Corps, Robinson et al. 2012), which draws a parallel with our strain data.

However, Jelinsky et al reported no significant change in ADAMTS genes in chronic tendinopathy (Jelinsky, Rodeo et al. 2011). Another study reported ADAMTS2, ADAMTS3 and ADAMTS5 were increased, increased and decreased respectively in painful tendon (Jones, Corps et al. 2006). ADAMTS4, ADAMTS7 and ADAMTS13 were increased, decreased and decreased respectively in ruptured tendon (Jones, Corps et al. 2006). We observed similar responses with strain in ADAMTS2 and ADAMTS4 but opposite effects in ADAMTS3, ADAMTS5, ADAMTS7 and ADAMTS13. We have shown an increase in ADAMTS10, ADAMTS14 and ADAMTS16, these were not regulated in either ruptured or painful tendon (Jones, Corps et al. 2006). We have shown an overall increase in ADAMTS mRNA expression with strain, however several ADAMTS genes were decreased in tendinopathy, again this indicates that the disease phenotype involves a complex set of causative factors, mechanical loading being one of them. TIMP3 expression at the mRNA level is lowered in both painful and ruptured tendon (Ireland, Harrall et al. 2001; Jones, Corps et al. 2006; Clegg, Strassburg et al. 2007). Here we show that TIMP3 is increased with strain, indicating a more anabolic phenotype compared to the disease state.

Both painful and ruptured Achilles tendon mRNA expression analysis showed decreased expression of the proteoglycan versican (Corps, Robinson et al. 2004; Karousou, Ronga et al. 2008), another study has shown an increase in expression of versican in Achilles tendinopathy (Ireland, Harrall et al. 2001). In painful and ruptured tendon increased aggrecan and biglycan mRNA is seen (Ireland, Harrall et al. 2001; Corps, Robinson et al. 2006; Clegg, Strassburg et al. 2007; Corps, Robinson et al. 2012). In chronic tendinosis or ruptured tendons a decrease (Alfredson, Lorentzon et al. 2003; Corps, Robinson et al. 2006) and an increase in ruptured tendon (Karousou, Ronga et al. 2008) have been reported in decorin mRNA. In the present study versican showed the opposite response to that seen in painful and ruptured Achilles tendon. However similar results were seen with loading compared to tendinopathy in aggrecan and biglycan, although changes were less marked and close to the cut off for significance. Collagen type I, type II and other glycoproteins such as fibronectin, and fibrillin were reported to increase in ruptured tendon and tendinopathies (Ireland, Harrall et al. 2001; Karousou, Ronga et al. 2008; Jelinsky, Rodeo et al. 2011; Corps, Robinson et al. 2012; Legerlotz, Jones et al. 2012), this is similar to the strain response. COMP was reported to decrease in tendinopathy (Jelinsky, Rodeo et al. 2011), whereas with strain we have shown an increase trend. No change was seen in

thrombospondin 1 mRNA expression in tendinopathy or ruptured tendon (Ireland, Harrall et al. 2001), whereas we have shown an increase in response to mechanical load, however this change is close to the cut off for significance.

IL6 was increased in both painful and ruptured Achilles tendon (6.3 and 180 fold respectively), however there was no change in painful posterior tibialis tendinopathy (Legerlotz, Jones et al. 2012). IL6R mRNA was decreased in ruptured as well as painful Achilles tendinopathy (Jelinsky, Rodeo et al. 2011; Legerlotz, Jones et al. 2012), however there was no significant change in painful posterior tibialis tendinopathy (Legerlotz, Jones et al. 2012). We have shown a trend to increase in IL6 and a significant decrease in IL6R in response to mechanical load, which is similar to the disease phenotype. An increase in IL6 mRNA with strain would suggest that IL6 signalling may be increased with mechanical load, however due to the fact that IL6R mRNA is decreased with strain this may not be the case. To confirm this measurement of proteins involved in canonical IL6 signalling is required (JAK/STAT). IL6 may however have some other function in response to strain other than canonical IL6 signalling.

COX2 is an inflammatory enzyme involved in the processing of prostaglandins. COX2 was increased in both painful and ruptured Achilles tendon (2 and 11 fold respectively), however there was no change in painful posterior tibialis tendinopathy (Legerlotz, Jones et al. 2012). We see a trend to increase in COX2 in response to mechanical loading, similar to the levels seen in painful tendinopathy, indicating that inflammatory mediators may be involved in the strain response. IGF is a growth factor involved in the stimulation of collagen expression (Gillery, Leperre et al. 1992). In the ligament one study has shown up to a 5 fold increase in IGF1 mRNA in response to rupture in a rabbit model (Sciore, Boykiw et al. 1998). This draws parallel with the 2 fold increase in IGF1 mRNA following 24 hours cyclic mechanical loading. As previously described in the introduction, TGFβ protein is increased in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002). However, we have shown little regulation of TGFβ mRNA in response to strain, further characterisation to confirm whether protein levels are effected by load are required.

Studies of tendinopathy have looked at the expression of ‘tendon markers’ to determine whether tenocytes have deviated from the tendon phenotype. Tenascin C mRNA was increased 2-4 fold in both tendinopathy and ruptured tendon (Ireland, Harrall et al. 2001; Jelinsky, Rodeo et al. 2011). An increase of more than 10 fold was seen in Tenascin C

mRNA in acute tendinopathy compared to adult controls in horse tendon (Taylor, Vaughan-Thomas et al. 2009), and a study of human tendon degeneration showed an increase in the levels of 300kd tenascin C protein, a large isoform of tenascin C generated by alternate splicing (Riley, Harrall et al. 1996). These changes coordinate with the changes in mRNA measured in the current study. Scleraxis was not differentially regulated in either acute or chronic tendon disease according to a study by Taylor and colleagues of equine tendon (Taylor, Vaughan-Thomas et al. 2009). A murine model of patella tendon injury measured an increase in scleraxis after 4-8 weeks, which returned to normal after 12 weeks (Scott, Sampaio et al. 2010). Therefore a temporal change in scleraxis may be involved in tendinopathy. This mirrors the increase seen in response to mechanical load in our study. An immunohistochemistry study showed the increased expression of SOX9 protein in patellar tendinopathy, with a more rounded cell phenotype suggesting a divergence to a cartilaginous phenotype (Rui, Lui et al. 2011). We have not observed any significant changes in SOX9 expression in response to loading. Therefore phenotypic divergence of tenocytes may have some involvement in the development of tendinopathy, perhaps in the absence of 'normal' loading. We have shown an increase in the 'tendon markers' in response to mechanical load, this indicates that tenocytes exposed to load may become more tendon like. However this is difficult to confirm due to the absence of definitive tendon markers.

Taking into account change in metalloproteinases, matrix genes, cytokines and tendon 'markers' in response to mechanical load and tendinopathy, we can see that there are a number of similarities between the two conditions, this is consistent with mechanical load playing role in tendinopathy. However, a more complex relationship seems to be involved in the disease phenotype, for example different magnitudes and frequencies of strain may induce differential regulation of key regulators of tendon stability. Differences between strain regulated and tendinopathic changes highlight the complex mechanisms of tendinopathy development and the limitations of using strain induced tendinopathy models. The homogeneous nature of tendinopathies is emphasized by the different gene expression patterns in tendinopathies. For example different tendon positions can influence the response to mechanical load, Legerlotz *et al* showed differential responses to mechanical load in Achilles compared to posterior tibialis tendon in a range of IL6 related genes (Legerlotz, Jones et al. 2012). Subsets of the disease or disease affecting tendon of

particular locations may involve different causative factors, however mechanical load seems to be the common denominator.

4.5. CONCLUSIONS

1. There is a general anabolic response to 5% mechanical loading at 1Hz in tenocytes derived from tendinopathic tendon.
2. The response to mechanical load is cell density dependant
3. Our investigation covers a larger range of genes than previously described in terms of mechanical regulation in human tenocytes. We have shown that MMP1, MMP2, MMP3, MMP13, ADAMTS2, ADAMTS4, ADAMTS10, Decorin, IL6, Lumican and Fibulin are decreased, and ADAM12, TIMP3, IGF1, Thrombospondin 1, COL1A1 and Elastin are increased in response to mechanical strain.
4. Similar patterns of regulation are seen in cells derived from normal and ruptured tendon.

CHAPTER 5: TGFβ and mechanical strain regulation of metalloproteinase and matrix genes

5.1. INTRODUCTION

As we have discussed in the introduction MMPs, matrix genes, transcription factors and cytokines are differentially regulated in tendinopathic tendon compared to normal tendon. As mechanical load is thought to play a key role in the development of tendinopathy, loading changes may be the cause of this differential regulation. Further characterisation of these changes may highlight key players in mechanotransduction. TGFβ is one such candidate, with an up regulation of protein expression in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002) and an up regulation of protein and mRNA with mechanical strain in tenocytes (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004) and other cell types (Riser, Cortes et al. 1996; Riser, Ladson-Wofford et al. 1999). However, studies have not looked at the activation status of TGFβ as well as the mRNA and total protein level in response to load.

The aims of this chapter are to elucidate the effects of TGFβ in the regulation of matrix metalloproteinase expression and to assesss whether TGFβ plays a role in mechanoregulation of metalloproteinase and matrix genes. To do this we compared the effects of TGFβ to the strain induced response in the Flexcell^{TM} 3D culture system. In addition we used a TGFβRI inhibitor (SB431542) which specifically inhibits the downstream phosphorylation of SMAD (Callahan, Burgess et al. 2002) to assesss whether TGFβ signalling is involved in the strain mediated regulation of metalloproteinase and matrix genes. We also measured levels of total and active TGFβ in the medium, using a cell based luciferase assay. We hypothesise that TGFβ regulates metalloproteinase and matrix genes in a similar (anabolic) way to mechanical loading and that TGFβ signalling is involved in the strain mediated regulation of metalloproteinase and matrix genes.

5.2. Methods

Analysis of gene expression with mechanical load: Tenocytes derived from tendinopathic, normal or ruptured tendon were seeded in type I collagen into Flexcell™ tissue train plates and loaded at 1Hz at 5% strain for a range of time points; 2, 4, 8, 24 and 48 hours at 1.5 x 10^6 cells/ml with or without TGFβ (1ng/ml). Gels were dissolved in Trizol and RNA was extracted using the tri-spin method essentially as described elsewhere (Ireland, Harrall et al. 2001). RNA was quantitated, reverse transcribed and analysed via qRT-PCR (Taqman) for a range of metalloproteinase and matrix genes (see table 2.1 in methods section). Data was normalised to the housekeeping gene TOP1 and expressed as the $2^{\Delta\Delta Ct}$. Statistical analysis was performed using the Wilcoxon signed rank test after a normality test (Shapiro Wilk W) confirmed the distribution was not normal. For full details see methods section 2.2 and 2.3.

Taqman low density array (TLDA) analysis: Due to the more robust response in cultures seeded at the higher cell density tenocytes were seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen (1mg/ml) were plated into tissue train Flexcell™ plates and uniaxially strained at 5% at 1Hz for up to 48 hours with or without TGFβ (1ng/ml) (Metalloproteinase and TIMP family analysis focused on 24 and 48 hours, whereas Time course analysis of selected genes looked at a time course of 2, 4, 8, 24 and 48 hours). Gels were harvested in Trizol and RNA extracted using the tri-spin method essentially as described elsewhere (Ireland, Harrall et al. 2001). RNA was quantitated, reverse transcribed and analysed using TLDA qRT-PCR. Metalloproteinase and TIMP family analysis focused 19 ADAMTS genes, all 23 MMP genes and all 4 TIMP genes. Genes were selected for analysis on the time course TLDA on the basis of robust response in the metalloproteinase and TIMP family TLDA (see table 2.2 and 2.3 in methods section for a full list of the genes analysed).

Data was normalised to 18s and expressed as the $2^{\Delta\Delta Ct}$. Statistical analysis was performed using the Student's t test (two tailed distribution, paired t test), as there were only 3 repeats we were unable to confirm whether these data were normally distributed and non-parametric tests are not suitable for n=3. For full details see methods section 2.3.4.

TGFβRI inhibition: Tenocytes seeded (1.5 x 10^6 cells/ml) in type I collagen gels (1mg/ml) were plated into Flexcell™ tissue train plates and uniaxially strained to 5% at

1Hz for 48 hours with or without TGFβRI inhibitor (SB431542, 10μM). Gels were harvested in Trizol and RNA extracted using the tri-spin method. RNA was quantitated, reverse transcribed and analysed using qRT-PCR for a selection of metalloproteinase and matrix genes. Data was normalised to TOP1 and expressed as the $2^{\Delta\Delta Ct}$. Statistical analysis was performed using the Wilcoxon signed rank test. For full details of protocols see methods section 2.3.

TGFβ activity measurement: TGFβ was measured using a cell based system targeting SMAD proteins involved in TGFβ signalling. Tenocytes seeded (1.5×10^6 cells/ml) in type I collagen gels (1mg/ml) were strained to 5% at 1Hz for 48 hours using the Flexcell™ vacuum powered system. Medium from 48 hour strained and non-strained gels was transferred onto SW1353 cells transfected with a SMAD specific construct linked to a luciferase reporter. Total TGFβ was measured by heat activating the TGFβ in the medium before incubation with the transfected SW1353 cells. Luciferin was added to samples and fluorescence measured on the luminometer. For details of this protocol see methods section 2.4.1.

5.3. RESULTS

5.3.1. Metalloproteinase and TIMP family analysis

The Metalloproteinase and TIMP family analysis TLDA covered 18 ADAMTS genes, all 23 MMP genes and all 4 TIMP genes analysing samples treated with or without strain or TGFβ at 24 and 48 hours. Of the 19 ADAMTS genes and 23 MMP genes the following were not sufficiently detected; ADAMTS8, ADAMTS15, ADAMTS18, ADMATS19, MMP12, MMP20, MMP21, MMP25, MMP26 and MMP28 (Average Ct>37). Genes with low expression (Ct between 35 and 37) included ADAMTS9, ADAMTS16 and ADAMTS17, MMP1, MMP3, MMP7, MMP8, MMP10, MMP15, MMP24 and MMP28.

MMP regulation with strain and TGFβ

At 24 hours the following genes were decreased with TGFβ; MMP2 (1.3 fold), MMP8 (7.1 fold), MMP11 (2.1 fold) and MMP19 (1.9 fold). At 24 hours MMP15 (2.7 fold) and MMP24 (12.5 fold) were increased with TGFβ (figure 5.1) (Statistical p values are shown in Table 5.2). MMP10 (53.5 fold) and MMP24 (5.2 fold) were increased with strain and MMP16 (2.1 fold) and MMP24 (30 fold) were increased with a combined strain and TGFβ treatment at 24 hours. At 48 hours MMP1 (11.1 fold), MMP10 (50 fold) and MMP13 (3.7 fold) were decreased with TGFβ. MMP24 was increased with strain (35.2 fold), TGFβ (21.7 fold) and a combination of the two (55.4 fold) and MMP3 (33.3 fold), MMP10 (4 fold) and MMP17 (2 fold) were decreased with strain at 48 hours. Also at 48 hours MMP1 and MMP17 were decreased with a combination of strain and TGFβ (10 fold and 1.6 fold respectively). TGFβ and strain responses were similar in pattern although each gene was not always significantly different (from controls) in both conditions.

ADAMTS regulation with strain and TGFβ

At 24 hours ADAMTS2 (2.1-2.9 fold), ADAMTS4 (3.2-9.9 fold), ADAMTS6 (3.6-5.8 fold), ADAMTS14 (2.7-4.9 fold) and ADAMTS16 (26-199 fold) were increased with strain, TGFβ and a combination of the two (Figure 5.2). ADAMTS10 was increased with TGFβ (2.4 fold). At 48 hours ADAMTS2 (1.7-2 fold), ADAMTS4 (7.1-10.2 fold) and ADAMTS16 (24.1-86 fold) were regulated with strain, TGFβ and a combination of the two. ADAMTS6 was significantly increased with strain (3.1 fold) and a combination of strain and TGFβ (2.4 fold). At 48 hours ADAMTS10 was increased with strain (2.6 fold)

and a combination of TGFβ and strain (2.7 fold). ADAMTS1 was decreased with a combination of strain and TGFβ (4.2 fold). ADAMTS12 (1.2-2.8 fold) and ADAMTS13 (1.7-2.9 fold) were decreased with TGFβ at 48 hours. The majority of ADAMTS responses were similar between strain and TGFβ.

TIMP regulation with strain and TGFβ

At 24 hours TIMP2 was decreased with TGFβ (1.3 fold), however there was little regulation with strain (figure 5.3). At 24 and 48 hours TIMP3 was similarly regulated with TGFβ (6.4 fold, 2.6 fold) and strain (4.6 fold, 5.3 fold) and a combination of the two (9 fold, 2.9 fold). At 48 hours TIMP1 and TIMP2 were decreased with a combination of TGFβ and strain (1.4 fold). TIMP4 was increased with TGFβ (1.5 fold), however there was no change with strain. There was poor regulation of TIMPs 1, 2 and 4 with strain alone.

The majority of MMPS, ADAMTSs and TIMPs were similarly regulated with strain and TGFβ. However ADAMTS5, ADAMTS7, MMP7, MMP15, MMP16 and TIMP4 are regulated by strain differently to TGFβ at either 24 or 48 hours (See fold changes outlined in bold, Table 5.1). ADAMTS5 and ADAMTS7 were decreased with TGFβ (1.4 and 1.5 fold respectively) and increased with strain (5.7 and 1.3 fold respectively) at 24 hours. At either 24 or 48 hours MMP15 and TIMP4 were increased with TGF (2.7 and 1.4 fold respectively) and with strain MMP15 remained unaffected (1 fold) and TIMP4 was decreased with strain (2.2 fold). MMP7 and MMP16 were decreased (1.4 and 1.4 fold respectively) with TGFβ and increased with strain (1.4 and 1.4 fold respectively) at 48 hours. TIMP4 was increased with TGFβ (1.5 fold) but was unaffected by mechanical loading at 48 hours.

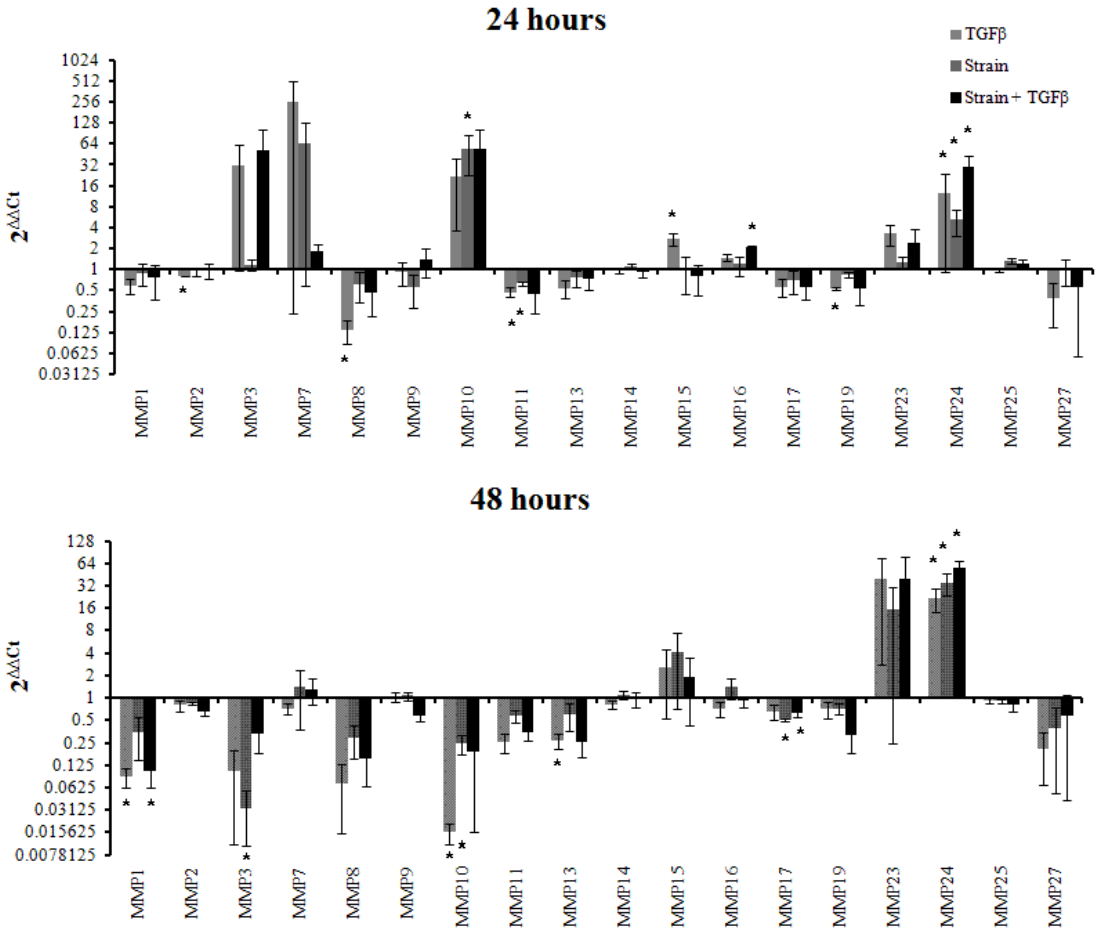

Figure 5.1. Metalloproteinase family expression in response to mechanical load and TGFβ: **TLDA analysis at 24 and 48 hours**. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over 24 and 48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * according to the paired t test ($p<0.05$).

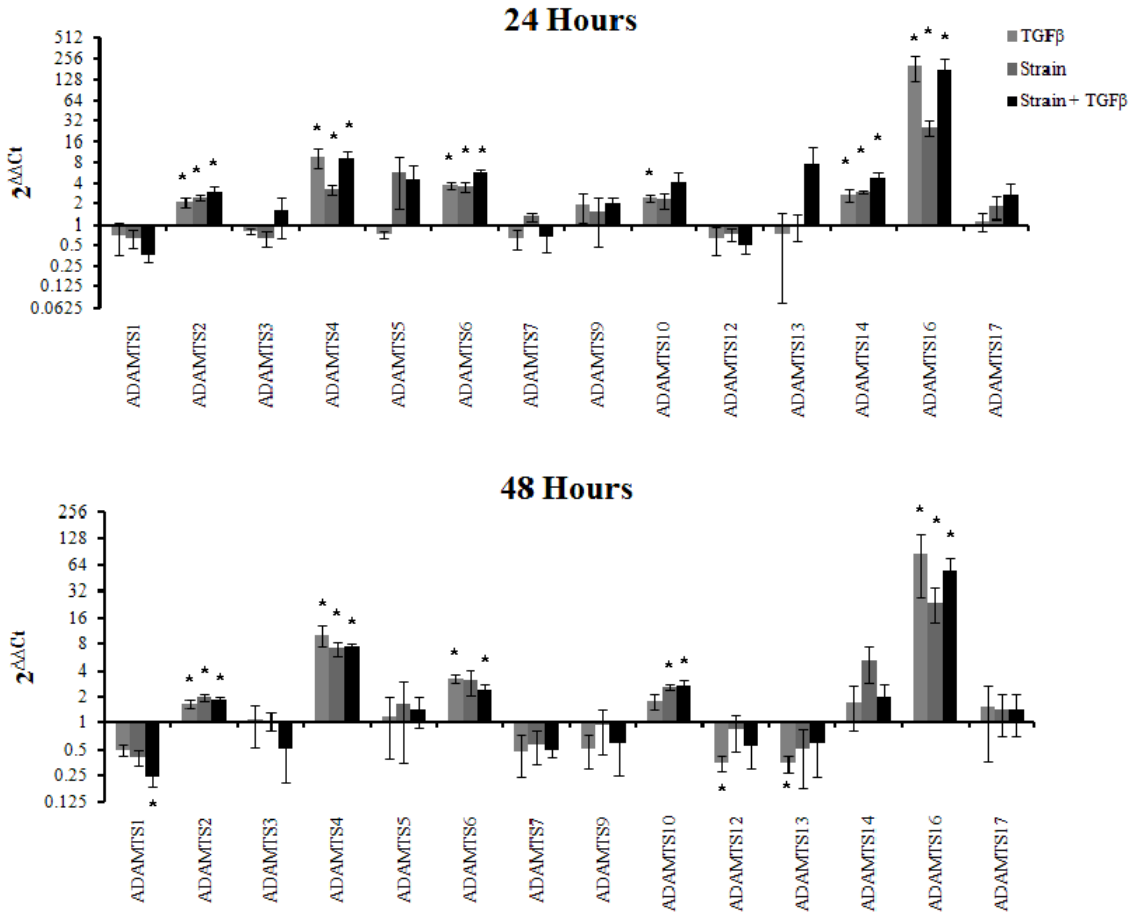

Figure 5.2. Regulation of ADAMTS family genes with mechanical loading and TGFβ: 24 and 48 hours. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over 24 and 48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * according to the paired t test ($p<0.05$).

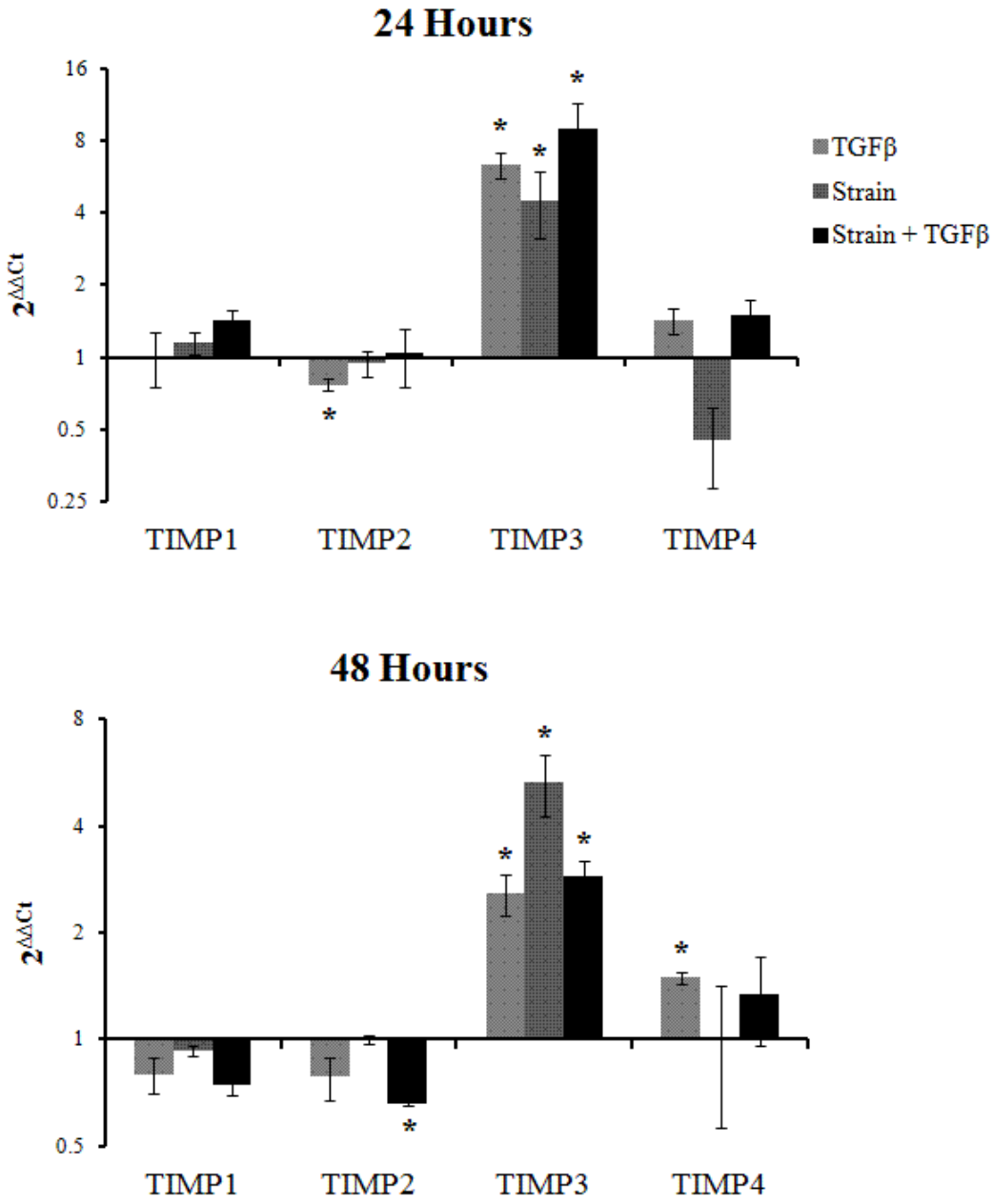

Figure 5.3. Regulation of TIMP family genes with mechanical loading and TGFβ: 24 and 48 hours. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over 24 and 48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * according to the paired t test ($p<0.05$).

	24 hours			48 hours			Average Ct
	TGFβ	Strain	Strain + TGFβ	TGFβ	Strain	Strain + TGFβ	
ADAMTS1	0.72	0.66	0.36	0.50	0.41	0.24	33
ADAMTS2	2.13	2.46	2.91	1.67	1.97	1.84	30
ADAMTS3	0.82	0.65	1.57	1.08	1.06	0.52	34
ADAMTS4	9.91	3.24	9.44	10.15	7.10	7.34	31
ADAMTS5	0.74	5.72	4.56	1.19	1.68	1.43	34
ADAMTS6	3.75	3.59	5.76	3.24	3.10	2.41	32
ADAMTS7	0.65	1.30	0.68	0.49	0.58	0.50	33
ADAMTS9	1.97	1.49	2.00	0.52	0.95	0.60	36
ADAMTS10	2.42	2.29	4.08	1.79	2.63	2.71	31
ADAMTS12	0.64	0.74	0.51	0.36	0.85	0.57	32
ADAMTS13	0.76	0.99	7.78	0.35	0.51	0.60	34
ADAMTS14	2.72	2.99	4.88	1.74	5.19	1.98	33
ADAMTS16	199.01	25.63	171.10	86.03	24.10	54.80	35
ADAMTS17	1.14	1.88	2.68	1.53	1.42	1.44	35
MMP1	0.58	0.89	0.77	0.09	0.35	0.10	36
MMP2	0.80	0.95	0.97	0.79	0.84	0.65	23
MMP3	31.69	1.17	51.88	0.10	0.03	0.33	37
MMP7	257.64	64.52	1.85	0.71	1.39	1.31	35
MMP8	0.14	0.62	0.46	0.07	0.29	0.15	36
MMP9	0.93	0.56	1.39	1.03	1.07	0.56	33
MMP10	21.85	53.52	53.73	0.02	0.25	0.19	37
MMP11	0.47	0.63	0.44	0.26	0.57	0.35	32
MMP13	0.54	0.76	0.75	0.27	0.60	0.25	31
MMP14	0.99	1.11	0.91	0.83	1.10	0.96	25
MMP15	2.71	0.99	0.80	2.53	4.16	1.92	35
MMP16	1.47	1.19	2.12	0.72	1.40	0.90	34
MMP17	0.57	0.71	0.55	0.67	0.51	0.62	32
MMP19	0.53	0.83	0.54	0.72	0.73	0.32	33
MMP23	3.29	1.28	2.38	38.89	15.49	39.20	34
MMP24	12.51	5.16	29.84	21.68	35.20	55.35	36
MMP27	0.39	0.99	0.56	0.21	0.39	0.57	38
TIMP1	1.01	1.15	1.43	0.79	0.93	0.74	24
TIMP2	0.77	0.95	1.03	0.78	1.00	0.66	25
TIMP3	6.39	4.55	8.99	2.57	5.31	2.90	27
TIMP4	1.42	0.45	1.50	1.49	0.99	1.34	34

Table 5.1. Fold changes in Metalloproteinase and TIMP family analysis with mechanical loading and TGFβ: 24 and 48 hours. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over 24 and 48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Mean Ct values are shown in the right hand column; Ct values of more than 35 are highlighted in light grey. Genes that showed a differential response to strain and TGFβ are highlighted with a black border.

	24 hours			48 hours			Average Ct
	TGFβ	Strain	Strain + TGFβ	TGFβ	Strain	Strain + TGFβ	
ADAMTS1	0.3615	0.2216	0.0612	0.0641	0.0532	0.0293	33
ADAMTS2	0.0370	0.0114	0.0408	0.0634	0.0254	0.0279	30
ADAMTS3	0.1427	0.2365	0.8194	0.5379	0.9652	0.2942	34
ADAMTS4	0.0183	0.0221	0.0128	0.0181	0.0095	0.0012	31
ADAMTS5	0.0985	0.2049	0.2522	0.6910	0.5939	0.8294	34
ADAMTS6	0.0079	0.0185	0.0064	0.0102	0.1469	0.0464	32
ADAMTS7	0.2654	0.2282	0.3911	0.2080	0.2475	0.0554	33
ADAMTS9	0.4830	0.7651	0.1709	0.1744	0.5596	0.3248	36
ADAMTS10	0.0147	0.1127	0.0727	0.1144	0.0075	0.0289	31
ADAMTS12	0.2883	0.2959	0.0982	0.0276	0.4968	0.2174	32
ADAMTS13	0.3558	0.7241	0.6022	0.0348	0.4454	0.2901	34
ADAMTS14	0.0354	0.0044	0.0094	0.7546	0.0776	0.6240	33
ADAMTS16	0.0044	0.0047	0.0082	0.0365	0.0228	0.0076	35
ADAMTS17	0.9237	0.2765	0.2040	0.4065	0.3994	0.3992	35
MMP1	0.1397	0.4521	0.3392	0.0099	0.1712	0.0242	36
MMP2	0.0029	0.6273	0.6922	0.2354	0.0694	0.0500	23
MMP3	0.6951	0.6612	0.1880	0.0956	0.0390	0.0939	37
MMP7	0.8895	0.9163	0.2277	0.2244	0.5380	0.6962	35
MMP8	0.0395	0.2635	0.2354	0.0585	0.2153	0.1209	36
MMP9	0.5936	0.3063	0.9687	0.9915	0.6702	0.0674	33
MMP10	0.6760	0.0205	0.1706	0.0069	0.0309	0.1265	37
MMP11	0.0299	0.0098	0.1397	0.0669	0.1012	0.0556	32
MMP13	0.1208	0.3234	0.3440	0.0380	0.2668	0.0772	31
MMP14	0.8451	0.3778	0.5259	0.2688	0.5518	0.7248	25
MMP15	0.0420	0.5702	0.4894	0.8129	0.6765	0.8317	35
MMP16	0.0890	0.8899	0.0025	0.2688	0.6636	0.5147	34
MMP17	0.1581	0.2920	0.1439	0.1755	0.0067	0.0380	32
MMP19	0.0065	0.1745	0.2204	0.2860	0.2121	0.1783	33
MMP23	0.1273	0.4269	0.7203	0.1712	0.9921	0.4741	34
MMP24	0.4879	0.0489	0.0472	0.0097	0.0067	0.0044	36
MMP27	0.1697	0.6102	0.2769	0.0883	0.2084	0.2529	38
TIMP1	0.7853	0.3687	0.0547	0.1838	0.1554	0.0449	24
TIMP2	0.0426	0.5946	0.8940	0.1887	0.9283	0.0007	25
TIMP3	0.0003	0.0229	0.0085	0.0177	0.0099	0.0082	27
TIMP4	0.1305	0.2016	0.1521	0.0053	0.6756	0.6512	34

Table 5.2. Statistical analysis with Metalloproteinase and TIMP family analysis with mechanical loading and TGFβ: 24 and 48 hours. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over 24 and 48 hours. 18s normalised data was statistically analysed using the students t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red. Mean Ct values are shown in the right hand column; Ct values of more than 35 are highlighted in light grey.

5.3.2. Time course analysis of selected genes TLDA

The time course analysis of selected genes TLDA included time points 2, 4, 8, 24 and 48 hours and included a selected number of metalloproteinase genes as well as a range of matrix genes, cytokines and tendon markers. Of these genes MMP8, ADAMTS16, COL2A1, Tenomodulin, IL1β, IL4, IL17, TNF and metabotropic glutamate receptor (GRM) 5 and GRM6 were not sufficiently detected. MMP3 was the only gene that had low expression across the range of conditions (Ct>35)

ADAMTS RNA analysis

ADAMTS10 was the only ADAMTS to be significantly increased with strain (2.8 fold) and TGFβ (3.5 fold) at 24 hours. At 8 and 24 hours ADAMTS6 was increased with TGFβ (3.6 fold and 5.3 fold) and a combination of strain and TGFβ (4.1 fold, 7.3 fold). ADAMTS5 significantly increased with a combination of strain and TGFβ (3.1 fold) at 8 hours. Patterns of regulation with strain and TGFβ at 48 hours were very similar except for ADAMTS5, where the increase in response to TGFβ occurred at 4 hours was not maintained until 48 hours. At 48 hours ADAMTS10 was increased with TGFβ (2.6 fold). Generally ADAMTS genes showed an early response with TGFβ and a later response to strain although these changes reached similar levels at their peak.

MMP RNA analysis

MMP1, MMP2, MMP3, MMP8, MMP10 and MMP13 were selected for analysis of the time course experiments. MMP2 was significantly decreased with both mechanical load (1.3 fold) and TGFβ (1.6 fold), with a combination of strain and TGFβ there was a trend to decrease. The other MMPs showed a trend to increase with TGFβ and strain at 48 hours, however this was not significant. MMP10 on the other hand was not clearly regulated by TGFβ, but was increased with strain at 2 hours (1.2 fold).

TIMP RNA analysis

TIMP4 was omitted from TLDA analysis as standard qRT-PCR failed to produce consistent results. TIMP1 and TIMP2 were not significantly regulated with mechanical load, however TIMP2 was decreased with TGFβ (1.4 fold). TIMP3 on the other hand was increased with both strain (5.9 fold at 48 hours) and TGFβ (2.6 and 6.5 fold at 4 and 8 hours respectively) although the response to TGFβ is much earlier than the response to strain.

Collagen RNA analysis

COL1A1 was increased with TGFβ at 48 hours (2.25 fold). COL3A1 was not regulated with mechanical loading; however it was increased with TGFβ at 24 hours (1.9 fold). COL12A1 was not significantly regulated by either mechanical loading or TGFβ. COL14A1 was decreased with TGFβ at 24 hours (2 fold). The combined effect of strain and TGFβ was similar to that of TGFβ alone in COL1A1, COL3A1 and COL14A1.

Matrix protein RNA analysis

Aggrecan was decreased with loading at 4 hours (1.4 fold); however this was not maintained later in the time course. Biglycan was significantly increased at 48 hours with TGFβ (1.74 fold). Decorin was decreased with strain at 2 hours (1.3 fold). A combination of TGFβ and strain induced a significant decrease in decorin at 48 hours (2.6 fold) similar. Lumican was decreased significantly with both strain (1.4 fold) and TGFβ (1.6 fold) at 48 hours. Versican was decreased with strain at 8 hours (1.2 fold). Versican was increased with TGFβ at 48 hours (1.3 fold). Fibrillin-1 was increased with a combination of strain and TGFβ at 24 hours (1.9 fold), and although not significant responses to strain or TGFβ. COMP was significantly increased at 48 hours with TGFβ and combined conditions (3.9 and 5.1 fold respectively). Fibronectin-1 was increased at 24 hours with both TGFβ (2.8 fold) and combined strain and TGFβ (2.3 fold). Thrombospondin-1 was significantly increased with strain at 24 hours (8.6 fold), the combination of TGFβ and strain induced an increase at 8 hours (6 fold). With respect to the response to TGFβ appears to be an earlier response compared to that with strain.

Tendon 'marker' RNA analysis

Although there are no true 'markers' of tendon, 'markers' of cell phenotype were chosen to identify whether cells differentiated from the tendon phenotype, these were tenascin C, scleraxis and SOX9. Tenascin C was increased with TGFβ at 4 hours (1.4 fold). Scleraxis was increased with TGFβ at 8 and 48 hours (5.6 and 4.8 fold respectively); in combined conditions Scleraxis was increased at 8 and 24 hours (7.8 and 13.5 fold. SOX9 was not clearly regulated by strain or TGFβ.

Cytokine RNA analysis

COX2 was not significantly regulated with either strain or TGFβ. IL6 was increased with TGFβ at 8 hours (21.2 fold). IL6R was decreased at 48 hours with strain (4.2 fold), TGFβ (9 fold) and combined conditions (6.8 fold). Therefore strain and TGFβ showed a similar

response although the TGFβ response occurred earlier than the strain induced response. IGF1 was increased with strain (2.3 fold, 24 hours), TGFβ (13.6 fold, 24 hours) and combined conditions (7.9 fold, 8 hours), although the response to TGFβ was far more marked and earlier than the response to strain. CTGF was increased with TGFβ at 24 hours (10.3 fold) and with the combined condition at both 4 (48.7 fold) and 8 hours (118.5 fold). TGFβ was not regulated significantly with strain, however at 4 (1.4 fold), 8 (1.8 fold) and 24 hours (2 fold) there was an increase with TGFβ and at 8 hours with combined treatment (2.2 fold).

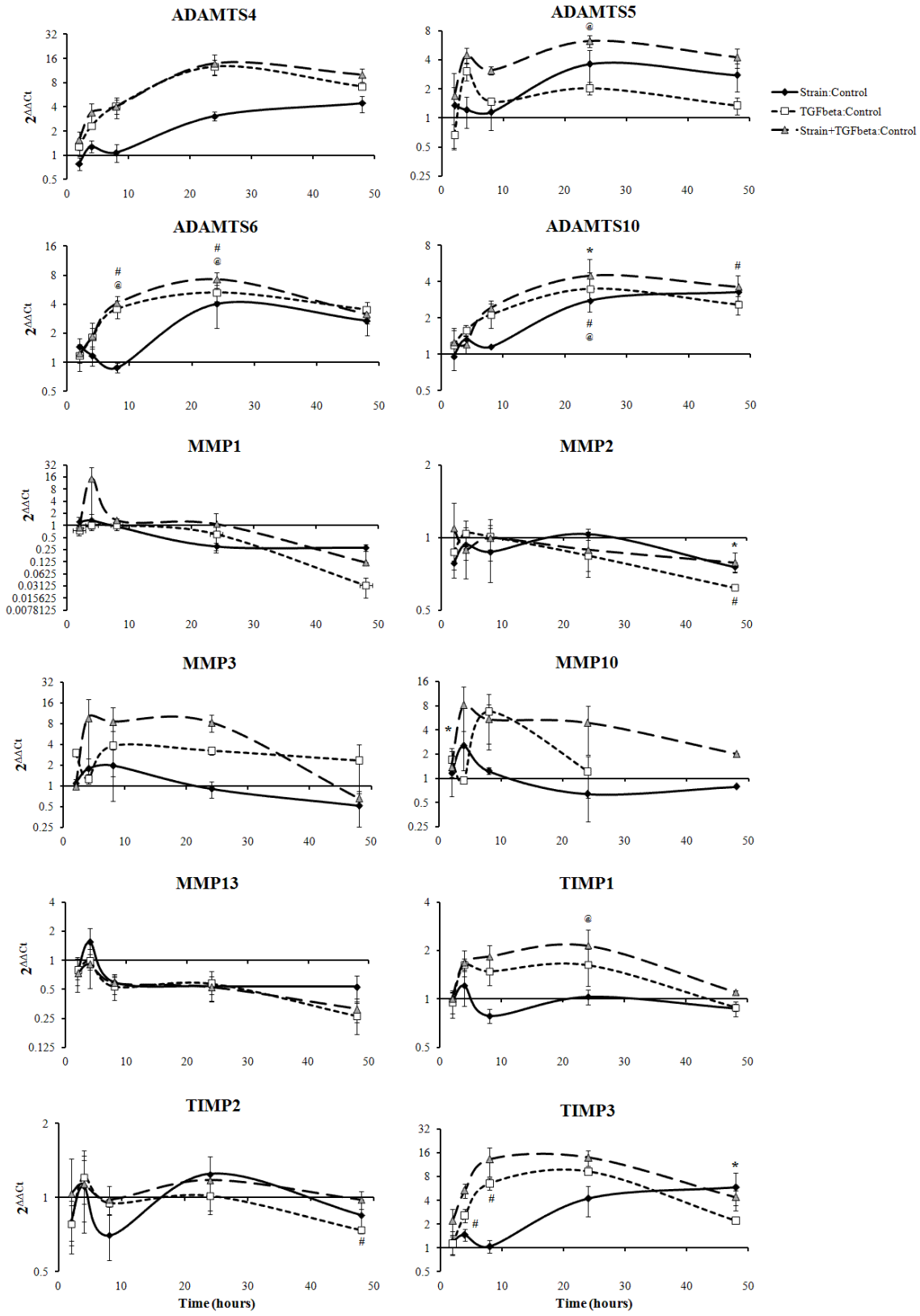

Figure 5.4. Regulation of metalloproteinases and TIMPs with mechanical loading: 0-48 hours. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * for strain, # for TGFβ and @ for strain plus TGFβ according to the paired t test ($p<0.05$).

		Time (hours)					Average Ct
		2	4	8	24	48	
ADAMTS4	Strain: Control	0.382	0.375	0.533	0.128	0.111	28
	TGFβ:Control	0.894	0.061	0.195	0.177	0.081	
	Strain+TGFβ:Control	0.320	0.244	0.266	0.119	0.164	
ADAMTS5	Strain: Control	0.718	0.561	0.883	0.088	0.172	33
	TGFβ:Control	0.227	0.141	0.225	0.150	0.326	
	Strain+TGFβ:Control	0.662	0.121	0.044	0.145	0.066	
ADAMTS6	Strain: Control	0.311	0.743	0.335	0.174	0.204	29
	TGFβ:Control	0.804	0.088	0.046	0.019	0.053	
	Strain+TGFβ:Control	0.565	0.322	0.030	0.042	0.081	
ADAMTS10	Strain: Control	0.702	0.385	0.642	0.047	0.153	29
	TGFβ:Control	0.860	0.094	0.138	0.044	0.014	
	Strain+TGFβ:Control	0.714	0.437	0.111	0.049	0.066	
MMP1	Strain: Control	0.712	0.580	0.811	0.120	0.637	32
	TGFβ:Control	0.406	0.697	1.000	0.251	0.417	
	Strain+TGFβ:Control	0.406	0.421	0.127	0.591	0.250	
MMP2	Strain: Control	0.273	0.726	0.381	0.585	0.031	20
	TGFβ:Control	0.428	0.706	0.705	0.394	0.003	
	Strain+TGFβ:Control	0.956	0.623	0.636	0.681	0.101	
MMP3	Strain: Control	0.894	0.842	0.666	0.328	0.399	36
	TGFβ:Control		0.434	0.315	0.164	0.813	
	Strain+TGFβ:Control		0.206	0.122	0.334	0.247	
MMP10	Strain: Control	0.023	0.507	0.235	0.382		35
	TGFβ:Control	0.552		0.084	0.963		
	Strain+TGFβ:Control	0.724	0.494	0.284	0.319		
MMP13	Strain: Control	0.227	0.599	0.304	0.357	0.372	29
	TGFβ:Control	0.659	0.467	0.160	0.405	0.289	
	Strain+TGFβ:Control	0.184	0.317	0.358	0.383	0.305	
TIMP1	Strain: Control	0.853	0.449	0.311	0.695	0.230	20
	TGFβ:Control	0.899	0.131	0.143	0.243	0.100	
	Strain+TGFβ:Control	0.722	0.262	0.019	0.282	0.097	
TIMP2	Strain: Control	0.269	0.956	0.193	0.356	0.421	21
	TGFβ:Control	0.354	0.635	0.320	0.954	0.022	
	Strain+TGFβ:Control	0.804	0.978	0.735	0.595	0.921	
TIMP3	Strain: Control	0.884	0.290	0.696	0.096	0.039	25
	TGFβ:Control	0.611	0.021	0.025	0.071	0.131	
	Strain+TGFβ:Control	0.292	0.085	0.066	0.070	0.104	

Table 5.3. Statistical analysis of metalloproteinases and TIMP regulation with mechanical loading and TGFβ: 0-48 hour time course TLDA. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. 18s Data normalised was statistically analysed using the students t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red. Mean Ct values are shown in the right hand column; Ct values of more than 35 are highlighted in light grey. Where no values appear some of the samples were undetectable

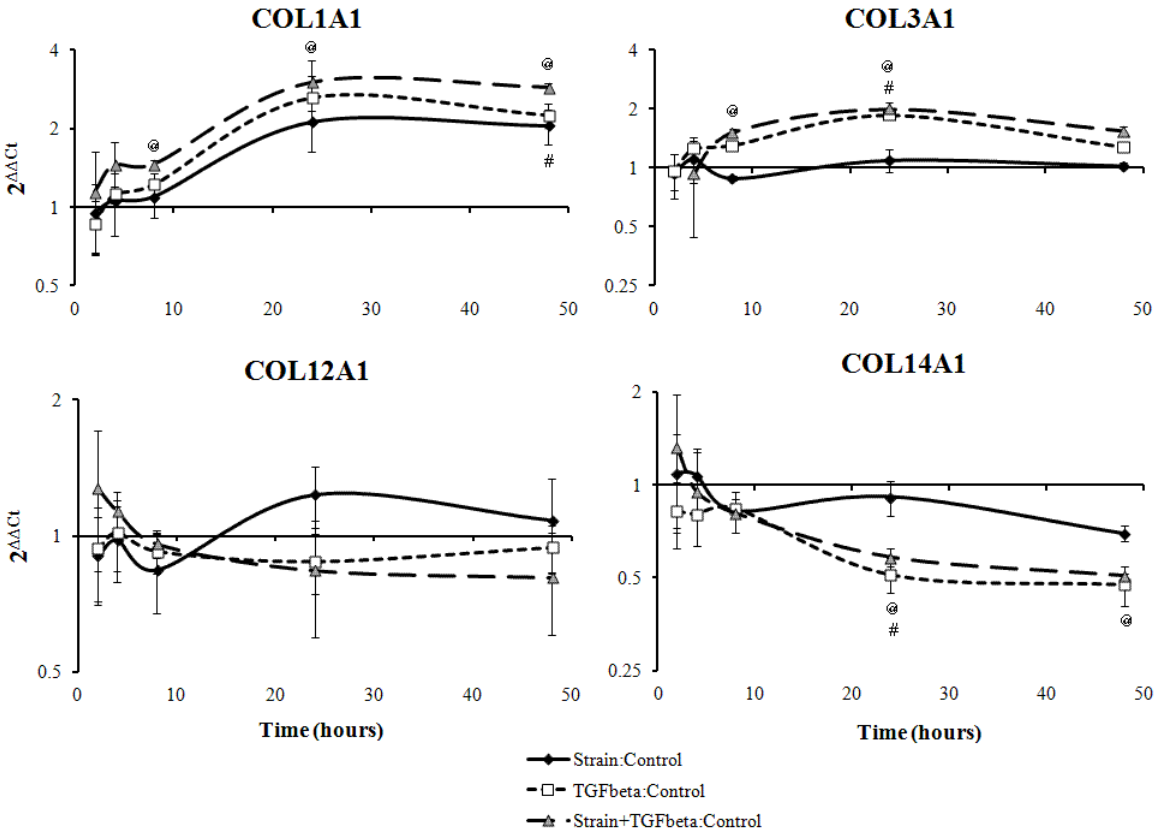

Figure 5.5. Regulation of Collagen expression with mechanical loading. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain ($2^{\Delta\Delta Ct}$). Significance is denoted by * for strain, # for TGFβ and @ for strain plus TGFβ according to the paired t test ($p<0.05$).

		Time (hours)					Average Ct
		2	4	8	24	48	
COL1A1	Strain: Control	0.400	0.811	0.473	0.190	0.129	20
	TGFβ:Control	0.390	0.382	0.200	0.122	0.018	
	Strain+TGFβ:Control	0.641	0.363	0.033	0.019	0.021	
COL3A1	Strain: Control	0.638	0.968	0.138	0.584	0.517	22
	TGFβ:Control		0.439	0.345	0.015	0.059	
	Strain+TGFβ:Control	0.752	0.814	0.024	0.013	0.056	
COL12A1	Strain: Control	0.322	0.642	0.500	0.405	0.836	24
	TGFβ:Control	0.604	0.654	0.544	0.407	0.600	
	Strain+TGFβ:Control	0.954	0.564	0.457	0.345	0.369	
COL14A1	Strain: Control	0.847	0.960	0.291	0.563	0.053	27
	TGFβ:Control	0.374	0.060	0.237	0.046	0.053	
	Strain+TGFβ:Control	0.900	0.680	0.054	0.040	0.019	

Table 5.4. Statistical analysis of collagen expression with mechanical loading and TGFβ: 0-48 hour time course TLDA. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. 18s normalised data was statistically analysed using the t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red. Mean Ct values are shown in the right hand column.

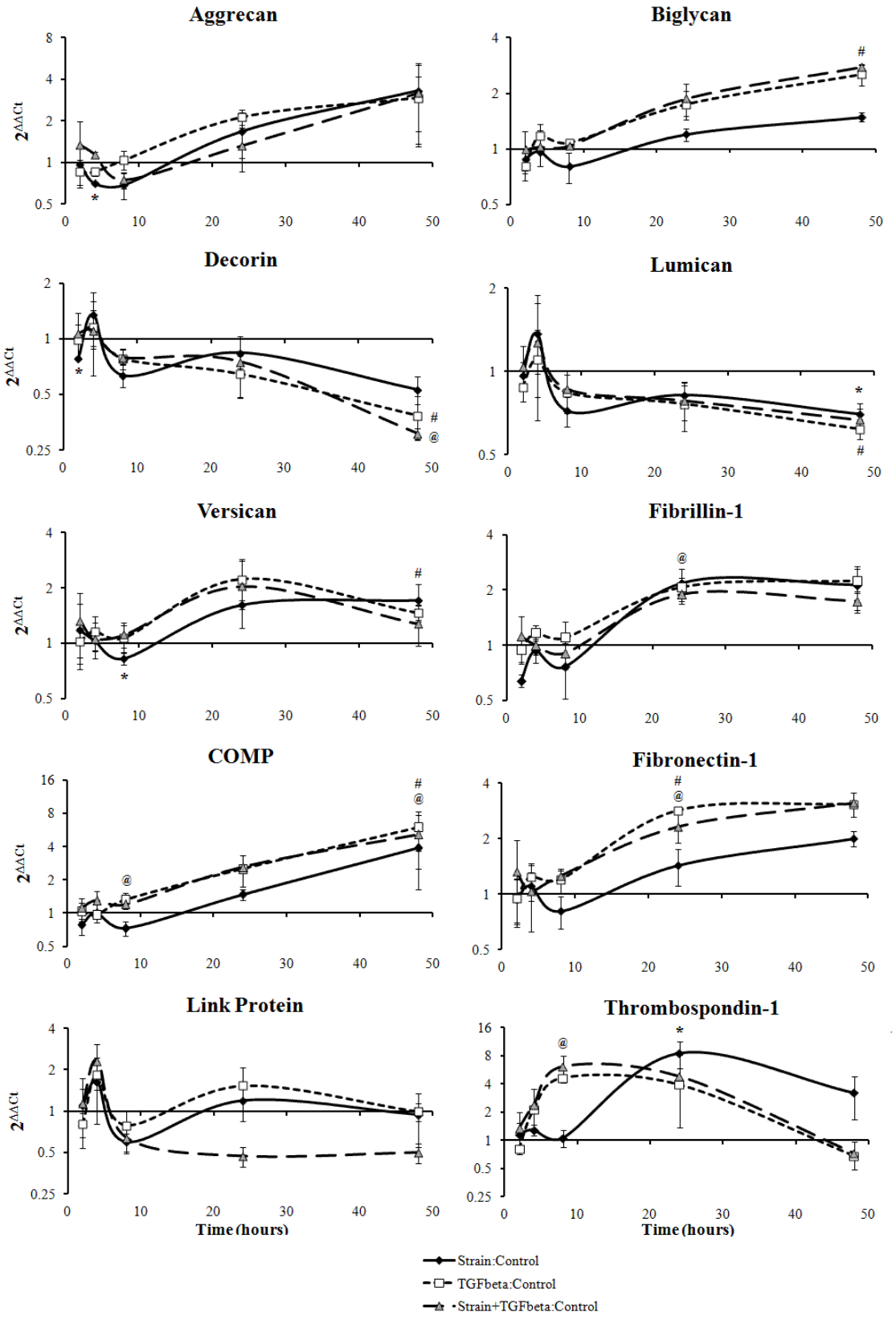

Figure 5.6. Regulation of Matrix gene expression with mechanical loading. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). Significance is denoted by * for strain, # for TGFβ and @ for strain plus TGFβ according to the students paired t test ($p<0.05$).

		Time (hours)					Average Ct
		2	4	8	24	48	
Aggrecan	Strain: Control	0.752	0.047	0.176	0.354	0.274	27
	TGFβ:Control	0.465	0.215	0.985	0.077	0.196	
	Strain+TGFβ:Control	0.828	0.154	0.169	0.602	0.365	
Biglycan	Strain: Control	0.290	0.586	0.392	0.250	0.099	21
	TGFβ:Control	0.301	0.644	0.130	0.121	0.007	
	Strain+TGFβ:Control	0.540	0.691	0.333	0.062	0.060	
Decorin	Strain: Control	0.019	0.527	0.175	0.434	0.059	26
	TGFβ:Control	0.930	0.635	0.279	0.209	0.045	
	Strain+TGFβ:Control	0.976	0.850	0.229	0.572	0.009	
Lumican	Strain: Control	0.933	0.623	0.117	0.188	0.021	25
	TGFβ:Control	0.313	0.994	0.356	0.275	0.000	
	Strain+TGFβ:Control	0.847	0.826	0.430	0.273	0.067	
Versican	Strain: Control	0.649	0.960	0.004	0.055	0.214	28
	TGFβ:Control	0.593	0.936	0.935	0.172	0.012	
	Strain+TGFβ:Control	0.785	0.568	0.607	0.212	0.819	
Fibrillin-1	Strain: Control	0.074	0.681	0.364	0.139	0.234	26
	TGFβ:Control	0.776	0.284	0.812	0.064	0.081	
	Strain+TGFβ:Control	0.960	0.673	0.480	0.018	0.083	
COMP	Strain: Control	0.369	0.659	0.249	0.058	0.098	26
	TGFβ:Control	0.980	0.348	0.305	0.246	0.016	
	Strain+TGFβ:Control	0.822	0.289	0.039	0.122	0.008	
Fibronectin	Strain: Control	0.441	0.648	0.364	0.292	0.062	18
	TGFβ:Control	0.501	0.341	0.540	0.027	0.068	
	Strain+TGFβ:Control	0.951	0.778	0.067	0.038	0.058	
Link Protein	Strain: Control	0.517	0.830	0.273	0.623	0.566	31
	TGFβ:Control	0.684	0.119		0.879	0.541	
	Strain+TGFβ:Control	0.551	0.608	0.258	0.283	0.215	
Thrombospondin 1	Strain: Control	0.812	0.070	0.388	0.045	0.295	23
	TGFβ:Control	0.211	0.070	0.123	0.137	0.083	
	Strain+TGFβ:Control	0.791	0.706	0.010	0.505	0.359	

Table 5.5. Statistical analysis of matrix gene regulation with mechanical loading and TGFβ: 0-48 hour time course TLDA. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. 18s Data normalised was statistically analysed using the t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red. Mean Ct values are shown in the right hand column; Ct values of more than 35 are highlighted in light grey. Where no values appear some of the samples were undetectable.

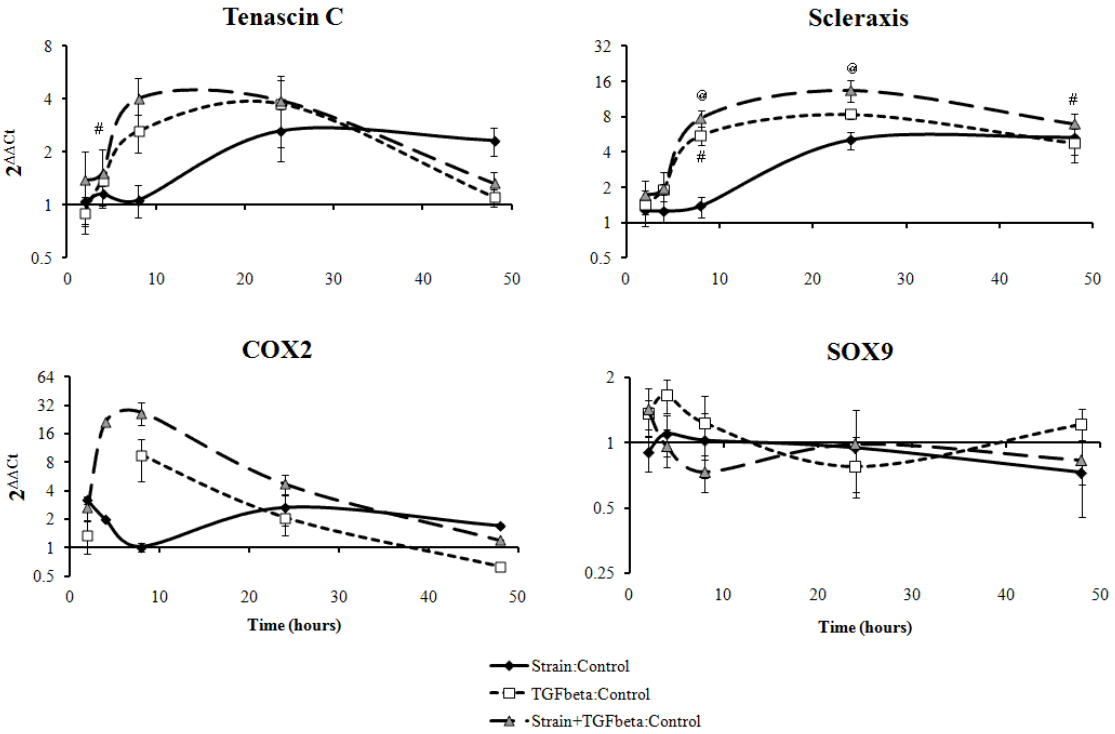

Figure 5.7. Regulation of cell lineage 'markers' with mechanical loading. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). Significance is denoted by * for strain, # for TGFβ and @ for strain plus TGFβ according to the paired t test ($p<0.05$).

		Time (hours)					Average Ct
		2	4	8	24	48	
Tenascin C	Strain: Control	0.556	0.615	0.594	0.242	0.236	23
	TGFβ:Control	0.559	0.043	0.158	0.181	0.197	
	Strain+TGFβ:Control	0.961	0.489	0.161	0.102	0.808	
Scleraxis	Strain: Control	0.573	0.632	0.190	0.167	0.115	25
	TGFβ:Control	0.563	0.172	0.032	0.056	0.009	
	Strain+TGFβ:Control	0.347	0.314	0.044	0.045	0.083	
COX2	Strain: Control	0.095		0.929	0.420	0.127	31
	TGFβ:Control	0.992		0.239	0.432		
	Strain+TGFβ:Control	0.192		0.100	0.183		
SOX9	Strain: Control	0.498	0.960	0.821	0.605	0.337	31
	TGFβ:Control	0.245	0.291	0.470	0.374	0.373	
	Strain+TGFβ:Control	0.562	0.628	0.250	0.843	0.667	

Table 5.6. Statistical analysis of cell lineage 'markers' regulation with mechanical loading and TGFβ: 0-48 hour time courseTLDA. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. 18s Data normalised was statistically analysed using the t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p>0.05$) are highlighted in red. Mean Ct values are shown in the right hand column, Ct values of more than 35 are highlighted in light grey, and those highlighted in darker grey were undetectable in the majority of samples. Where no values appear some of the samples were undetectable

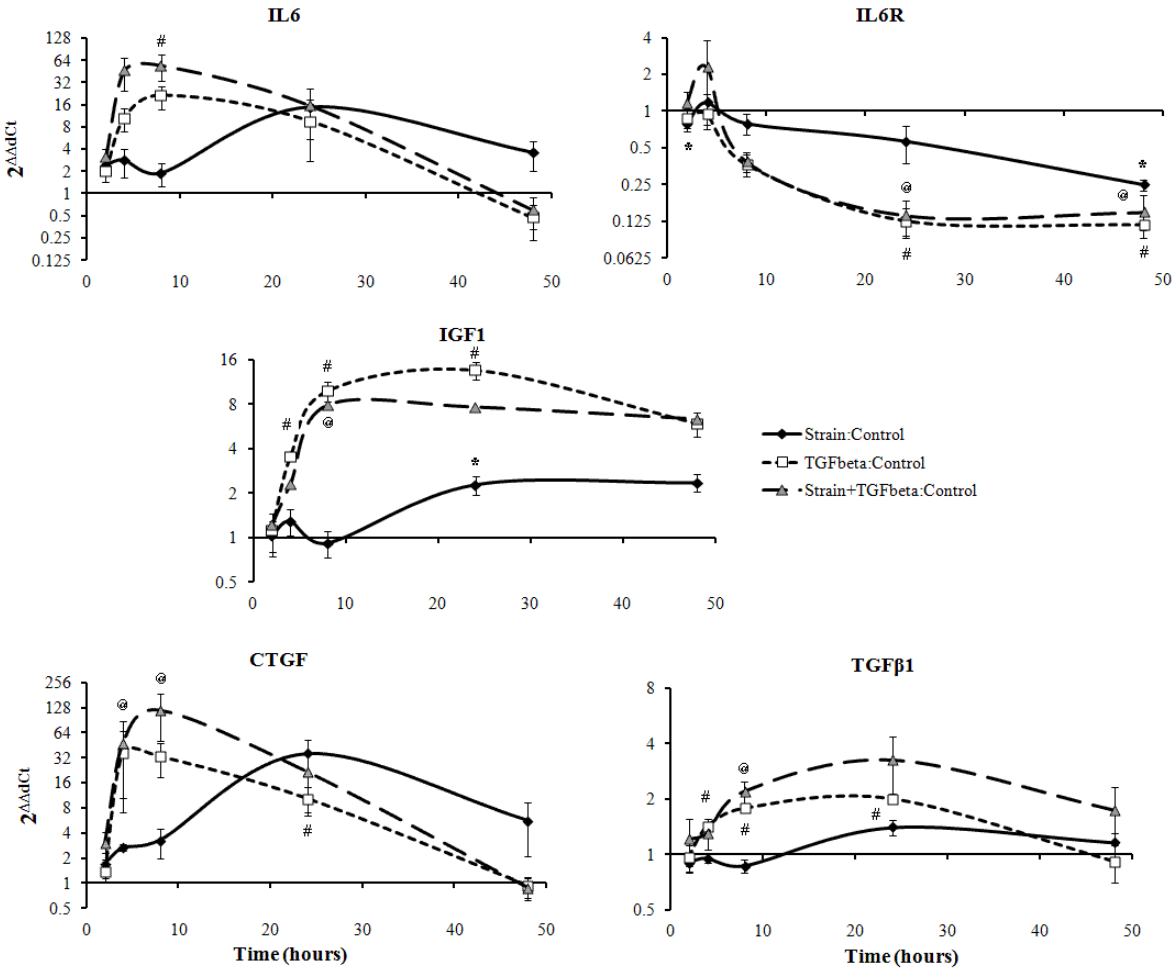

Figure 5.8. Regulation of cytokine expression with mechanical loading. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to 18s and presented as a mean (± SE, n=3) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). Significance is denoted by * for strain, # for TGFβ and @ for strain plus TGFβ according to the paired t test ($p<0.05$).

		Time (hours)					Average Ct
		2	4	8	24	48	
IL6	Strain: Control	0.055	0.215	0.208	0.169	0.346	31
	TGFβ:Control	0.179	0.065	0.003	0.213	0.441	
	Strain+TGFβ:Control	0.090	0.129	0.086	0.112	0.215	
IL6R	Strain: Control	0.020	0.801	0.256	0.092	0.036	31
	TGFβ:Control	0.515	0.560	0.125	0.025	0.039	
	Strain+TGFβ:Control	0.851	0.572	0.224	0.007	0.018	
IGF1	Strain: Control	0.860	0.377	0.719	0.041	0.228	27
	TGFβ:Control	0.678	0.027	0.015	0.026	0.060	
	Strain+TGFβ:Control	0.575	0.351	0.028	0.078	0.082	
CTGF	Strain: Control	0.305	0.164	0.333	0.089	0.301	24
	TGFβ:Control	0.871	0.166	0.198	0.022	0.695	
	Strain+TGFβ:Control	0.031	0.223	0.024	0.255	0.681	
TGFβ1	Strain: Control	0.446	0.468	0.287	0.237	0.524	23
	TGFβ:Control	0.815	0.038	0.009	0.006	0.584	
	Strain+TGFβ:Control	0.614	0.318	0.010	0.355	0.278	

Table 5.7. Statistical analysis cytokine regulation with mechanical load and TGFβ: 0-48 hour time course TLDA. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a period of 0-48 hours. 18s normalised data was statistically analysed using the students t-test (2-tailed, paired t-test) (n=3). Statistical p values are shown, those below the significance cut of point ($p>0.05$) are highlighted in red. Mean Ct values are shown in the right hand column; Ct values of more than 35 are highlighted in light grey.

5.3.3. Strain and TGFβ regulation of selected Metalloproteinase and matrix genes

A number of genes were selected for further qRT-PCR analysis. MMPs were chosen because of our particular interest in the regulation of matrix turnover with mechanical load and the anabolic effects seen in our preliminary TLDA. ADAM12 was chosen because of its response in tendinopathy and its regulation with mechanical load seen earlier. ADAMTS5 was chosen due to the differential effects of TGFβ and strain upon its expression in the preliminary analysis. COL1A1 and Elastin were chosen as they are the most abundant components of the tendon ECM. Fibulin-1 was chosen due to existing interests in the laboratory and its interaction with elastin.

MMP1 (48 hours), MMP2 (24 and 48 hours), MMP3 (4 hours) and fibulin-1 (48 hours) were significantly decreased with mechanical load, TGFβ and a combination of the two. Elastin was increased significantly with all three conditions at 8, 24 and 48 hours. MMP13 was significantly decreased with both strain and TGFβ at 24 and 48 hours. MMP3 is significantly decreased with TGFβ at 2 and 4 hours and decreased with strain after 48 hours. However at 24 hours TGFβ induces an increase in MMP3, which is not seen in response to strain. TGFβ treatment causes an increase in ADAM12 expression at 8, 24 and 48 hours, however the response to strain is only significant after 24 hours. MMP1 (4, 8 and 24 hours), MMP3 (2 and 4 hours) and fibulin-1(2, 4, 8 and 24 hours) responded to TGFβ at much earlier time points than they responded to mechanical load. COL1A1 was increased with TGFβ at 4, 8, 24 and 48 hours however there was only a significant response to strain at 24 hours. ADAMTS5 was different to the other genes, it was increased at 24 hours with strain or a combination of TGFβ and strain, however there were no significant changes with TGFβ alone.

Generally the combination of TGFβ and strain did not cause an additive effect accept for in Fibulin-1 where there was more of a decrease with a combination of the TGFβ and strain. Elastin on the other hand showed less of a response compared to TGFβ treatment at the earlier time points. Generally TGFβ responses were more marked compared to those responses to mechanical loading, especially in MMP1 and MMP13 at 48 hours, and in Elastin at around 8 hours.

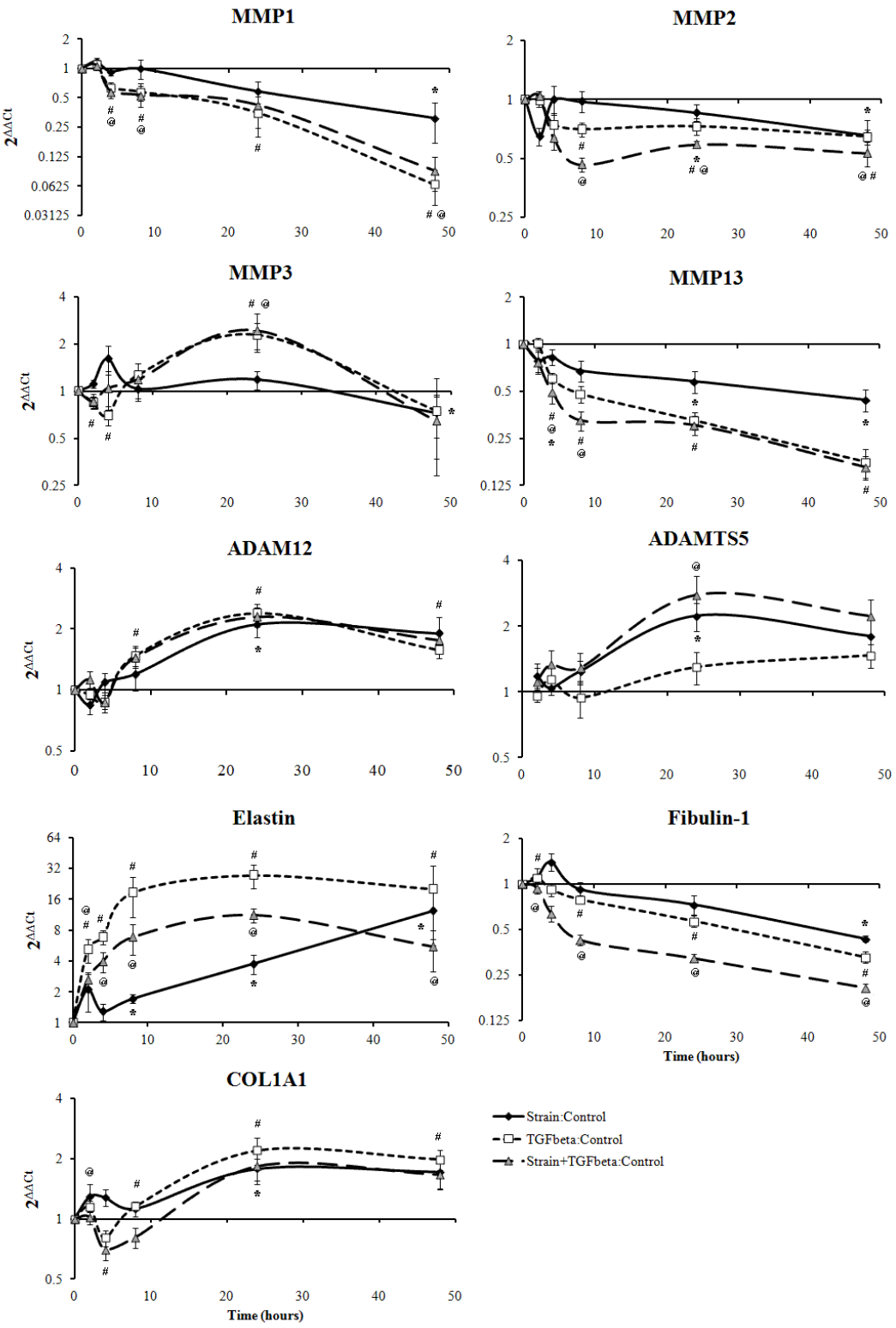

Figure 5.9. Strain and TGFβ modulation of selected Metalloproteinases and matrix genes. Standard quantitative Real Time PCR analysis of cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to TOP1 and presented as a mean (± SE, n=3) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). Significant values are indicated as * (changes with strain), # (changes with TGFβ) or @ (changes with strain plus TGFβ) according to the Wilcoxon signed rank test ($p<0.05$)

Time (hours)		MMP1	MMP2	MMP3	MMP13	ADAM12	ADAMTS1	Elastin	Fibulin-1	COL1A1
2	Strain	0.128	0.612	0.237	0.091	0.735	0.173	0.463	0.866	0.398
	TGFβ	1	1	0.028	0.735	1	0.463	0.028	0.028	0.063
	Strain + TGFβ	0.866	0.612	0.237	0.128	0.612	0.917	0.028	0.398	1
4	Strain	0.333	0.445	0.386	0.038	0.386	0.6	0.249	0.128	0.091
	TGFβ	0.018	0.063	0.043	0.018	0.128	0.917	0.028	0.176	0.018
	Strain + TGFβ	0.018	0.028	0.237	0.018	0.237	0.173	0.028	0.018	0.018
8	Strain	0.333	0.508	0.114	0.086	0.959	0.116	0.018	0.063	0.398
	TGFβ	0.018	0.018	0.499	0.018	0.028	0.753	0.018	0.028	0.018
	Strain + TGFβ	0.028	0.018	0.866	0.018	0.237	0.463	0.028	0.018	0.91
24	Strain	0.051	0.038	0.953	0.012	0.008	0.028	0.046	0.075	0.028
	TGFβ	0.046	0.046	0.046	0.028	0.028	0.249	0.028	0.028	0.028
	Strain + TGFβ	0.173	0.028	0.028	0.6	0.753	0.046	0.046	0.028	0.345
48	Strain	0.021	0.015	0.01	0.008	0.051	0.116	0.046	0.028	0.173
	TGFβ	0.028	0.028	0.345	0.028	0.028	0.075	0.028	0.028	0.028
	Strain + TGFβ	0.046	0.046	0.686	0.917	0.753	0.075	0.046	0.028	0.116

Table 5.8. Statistical analysis of Strain and TGFβ modulation of selected Metalloproteinases and matrix genes. Taqman Low density array analysis of cells seeded at 1.5 x 10^6 cells/ml in type I rat tail collagen at 1 mg/ml after 5% cyclic strain over a period of 0-48 hours. TOP1 normalised data was statistically analysed using Wilcoxon signed rank test. Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red ($n\geq6$).

5.3.4. TGFβRI abrogated strain induced gene regulation

In order to determine whether TGFβ signalling was involved in the mechanical regulation of the genes selected in figure 5.9, an inhibitor of TGFβRI was added to tenocyte seeded gels before 48 hours of mechanical strain. The TGFβRI inhibitor is an ATP binding site kinase inhibitor which has been shown to inhibit immobilised SMAD phosphorylation, therefore it effectively inhibits the phosphorylation of SMAD by TGFβRI (IC_{50} 94nM). Previous studies have used this inhibitor at 10μM and have shown no effect upon cell viability (Callahan, Burgess et al. 2002; Inman, Nicolas et al. 2002). We have not tested for cell viability, however no observable changes were seen in cell morphology and levels of RNA were consistent upon extraction from cultures. This inhibitor is specific for ALK4, ALK5 and ALK7. Previous publications have shown that the inhibitor does not affect other ALK receptors including those involved in BMP signalling (ALK1, ALK2, ALK3 or ALK6) (Callahan, Burgess et al. 2002; Inman, Nicolas et al. 2002).

MMP1, MMP3 and fibulin-1 were decreased with mechanical loading as we have seen earlier, however the addition of the TGFβRI inhibitor caused a significant increase in the expression of these genes and therefore caused an abrogation of the strain induced response. ADAM12, Elastin and COL1A1 were increased with mechanical loading and TGFβ as we have previously been reported, however with the addition of the TGFβRI inhibitor the expression of these genes was decreased. Again this means that there was an abrogation of the strain induced response. ADAMTS5, which was differentially regulated with mechanical loading in the previous experiment, did not show a decrease in the strain response with the addition of the TGFβRI inhibitor. In fact the TGFβRI inhibitor increased the strain induced effects. Even in the non-strained control cultures the addition of the inhibitor causes the mRNA level to change in the opposite direction to the strain response.

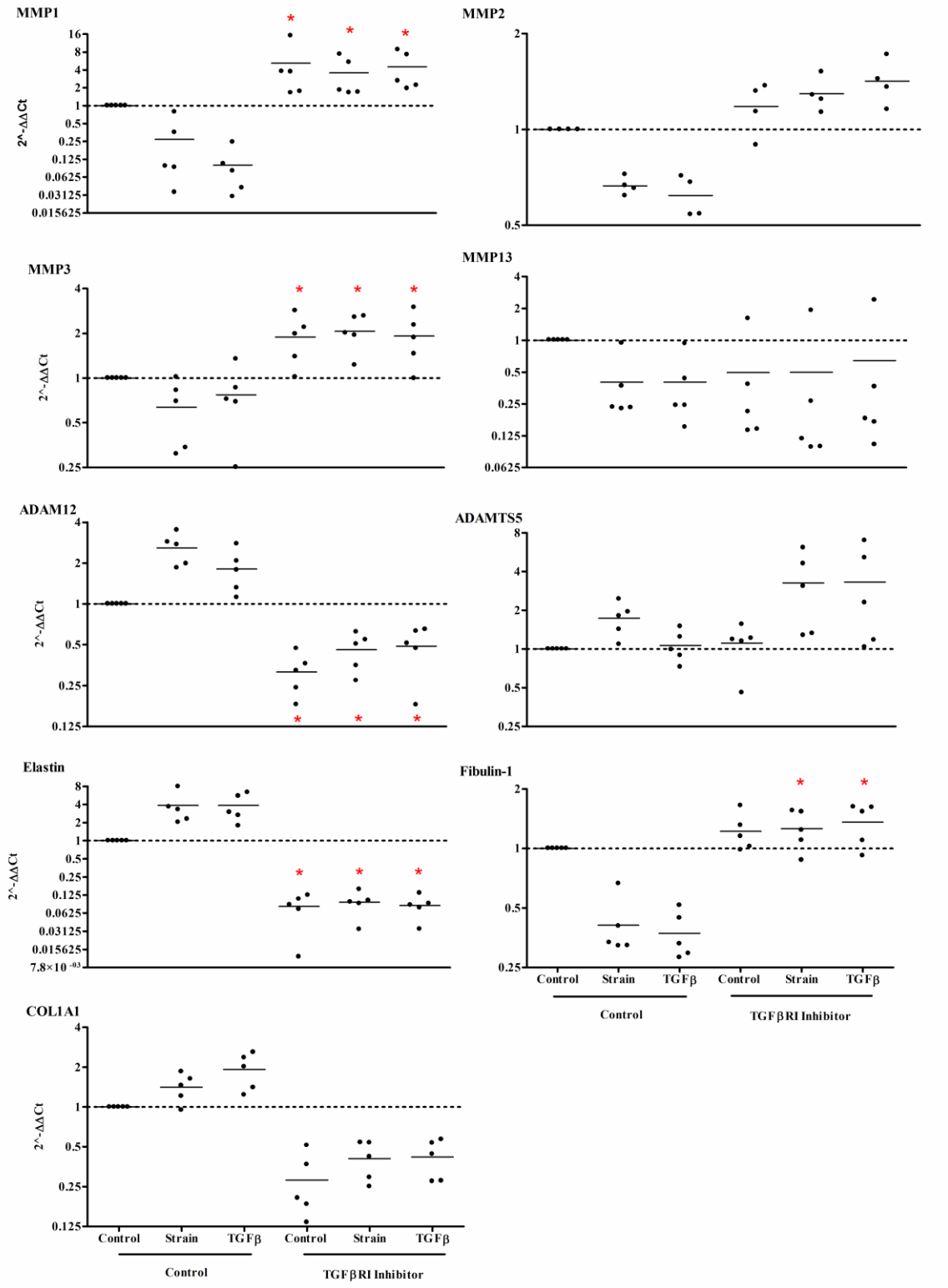

Figure 5.10. The effect of a TGFβRI inhibitor on strain regulated gene expression. Quantitative Real Time PCR analysis of cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml (+/- TGFβRI inhibitor, 10μM) after 5% cyclic strain at 48 hours. Data was normalised to TOP1 and presented as a mean (n=5) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). TOP1 normalised data was statistically analysed using Wilcoxon signed rank test. Values marked with * were significantly different from the same condition without TGFβRI inhibitor ($p<0.05$).

	Non-Strain: Strain	Strain: Strain + Inhibitor	Non-Strain: Non-strain + TGFβ	Non-strain TGFβ: Non-strain + TGFβ + Inhibitor	Non-strain: Non-strain + Inhibitor	Non-strain: Strain + Inhibitor
MMP1	0.043	0.043	0.043	0.043	0.043	0.043
MMP2	0.043	0.138	0.043	0.138	0.138	0.138
MMP3	0.08	0.043	0.08	0.043	0.043	0.043
MMP13	0.043	0.5	0.043	0.686	0.5	0.5
ADAM12	0.043	0.043	0.043	0.043	0.043	0.043
ADAMTS5	0.043	0.08	0.893	0.686	0.345	0.043
Elastin	0.043	0.043	0.043	0.043	0.043	0.043
Fibulin	0.043	0.043	0.043	0.043	0.345	0.138
COL1A1	0.08	0.043	0.043	0.043	0.043	0.043

Table 5.9. Statistical analysis of the effect of the TGFβRI inhibitor. Quantitative Real Time PCR analysis of cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml (+/- TGFβRI inhibitor, 10μM) after 5% cyclic strain at 48 hours. TOP1 normalised data was statistically analysed using Wilcoxon signed rank test. Statistical p values are shown, those below the significance cut of point ($p<0.05$) are highlighted in red (n=5).

5.3.5. TGFβ isoform and receptor regulation with mechanical load

There was no significant increase in TGFβ isoforms expression with strain at any of the time points that were analysed (see figure 5.11). TGFβ1 was decreased with mechanical load after 8 hours of loading (1.3 fold); TGFβ2 was also decreased with load after 24 hours (2.7 fold). TGFβ3 was decreased with strain after 8 and 24 hours (1.6 and 1.7 fold respectively). TGFβ1 and TGFβ2 were increased with TGFβ treatment after 2 hours (1.5 and 1.5 fold respectively), and TGFβ3 was decreased with TGFβ treatment after 4 and 8 hours (1.7 and 2.8 fold respectively). The combination of TGFβ and strain caused an additive effect with respect to TGFβ2 and TGFβ3 at 8 hours. On the other hand TGFβRI was increased with strain at 24 hours (2.1 fold). However, TGFβRI responded to TGFβ treatment at 2, 4 and 8 hours (1.6, 1.5 and 1.8 fold increase) much earlier than the response to strain. TGFβRI responded to a combination of TGFβ and strain in a similar manner to the response to TGFβ alone.

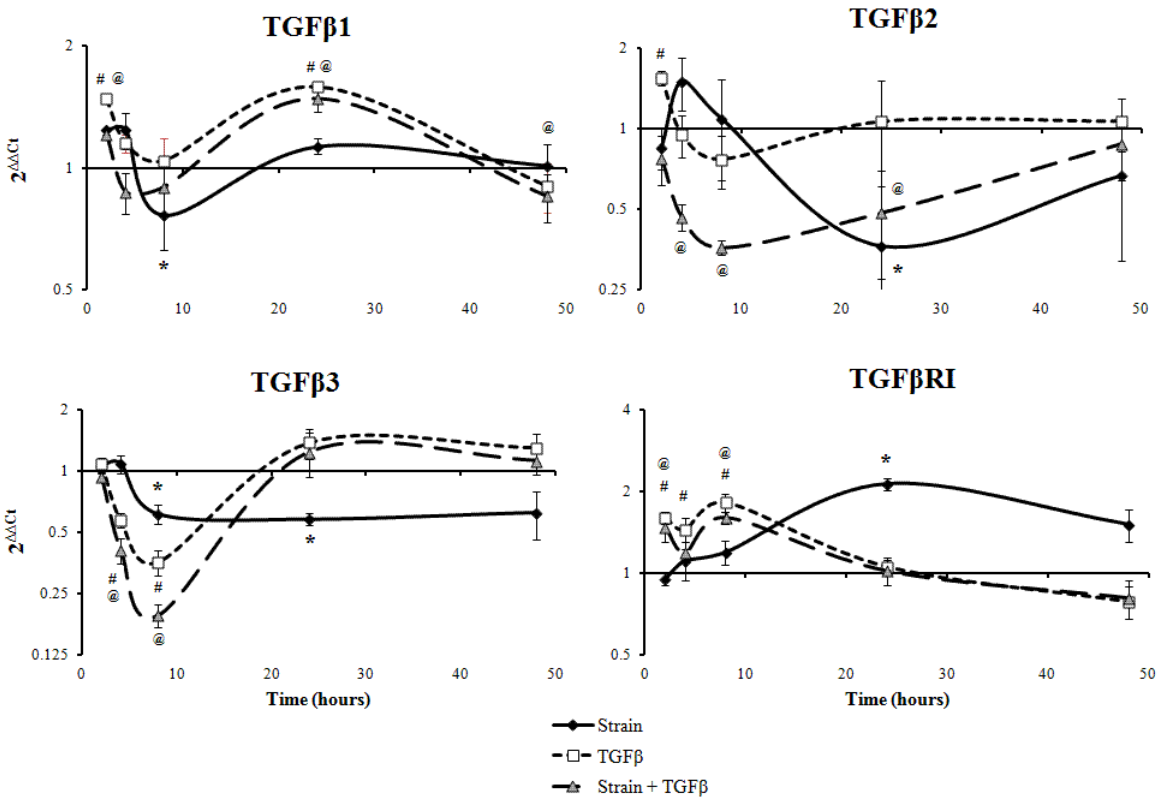

Figure 5.11. Regulation of TGFβ isoform and TGFβRI mRNA expression with strain and TGFβ. Quantitative Real Time PCR analysis of TGFβ isoforms in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data was normalised to TOP1 and presented as a mean (n=6, ± SE) fold change with strain and/or TGFβ ($2^{\Delta\Delta Ct}$). Significant values are indicated as * (strain: non-strain), # (control: TGFβ) or @ (control: Strain plus TGFβ) according to the Wilcoxon signed rank test ($p<0.05$).

TGFβ1

	2	4	8	24	48
Strain	0.173	0.075	0.028	0.345	0.917
TGFβ	0.028	0.463	0.917	0.028	0.116
Strain + TGFβ	0.046	0.249	0.116	0.028	0.046

TGFβ2

	2	4	8	24	48
Strain	0.173	0.463	0.249	0.028	0.249
TGFβ	0.028	0.753	0.345	0.463	0.345
Strain + TGFβ	0.249	0.028	0.028	0.046	0.249

TGFβ3

	2	4	8	24	48
Strain	0.6	0.345	0.028	0.028	0.249
TGFβ	0.249	0.028	0.028	0.116	0.249
Strain + TGFβ	0.173	0.028	0.028	0.753	0.6

TGFβRI

	2	4	8	24	48
Strain	0.249	0.345	0.173	0.028	0.116
TGFβ	0.028	0.046	0.028	0.463	0.249
Strain + TGFβ	0.028	0.753	0.028	0.6	0.173

Table 5.10. Statistical analysis of TGFβ isoform and TGFβRI regulation with strain and TGFβ. Quantitative Real Time PCR analysis of TGFβ isoforms in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain over a time course of 0-48 hours. Data normalised to TOP1 and expressed as a ratio of strain: non-strain, control: TGFβ and control: Strain plus TGFβ (n>6). The Wilcoxon signed rank test was used to test statistical significance ($p<0.05$). P values are shown above; significant values are highlighted in red.

5.3.6. Active TGFβ is increased with mechanical loading

Preliminary experiments over a time course of 2, 4, 8, 12, 24 and 48 hours show that active TGFβ in increased after 24 and 48 hours (Figure 5.12). At 48 hours TGFβ was similar between strained and non-strained cultures in terms of total TGFβ. However the level of active TGFβ was significantly increased in strained cultures compared to non-strain (~40 fold increase, p=0.028). This indicates that strain induces TGFβ activation.

A Pan TGFβ inhibitory antibody as well as the TGFβRI inhibitor inhibits the detection of SMAD activity in strain conditioned media from 3 cell isolates (See section 3.3 of the optimisation chapter). This indicates that the assay is specific to TGFβ and not BMP signalling. We have not added BMP to the assay to determine whether it detects BMP signalling. However, doing so would confirm the specificity of the assay further.

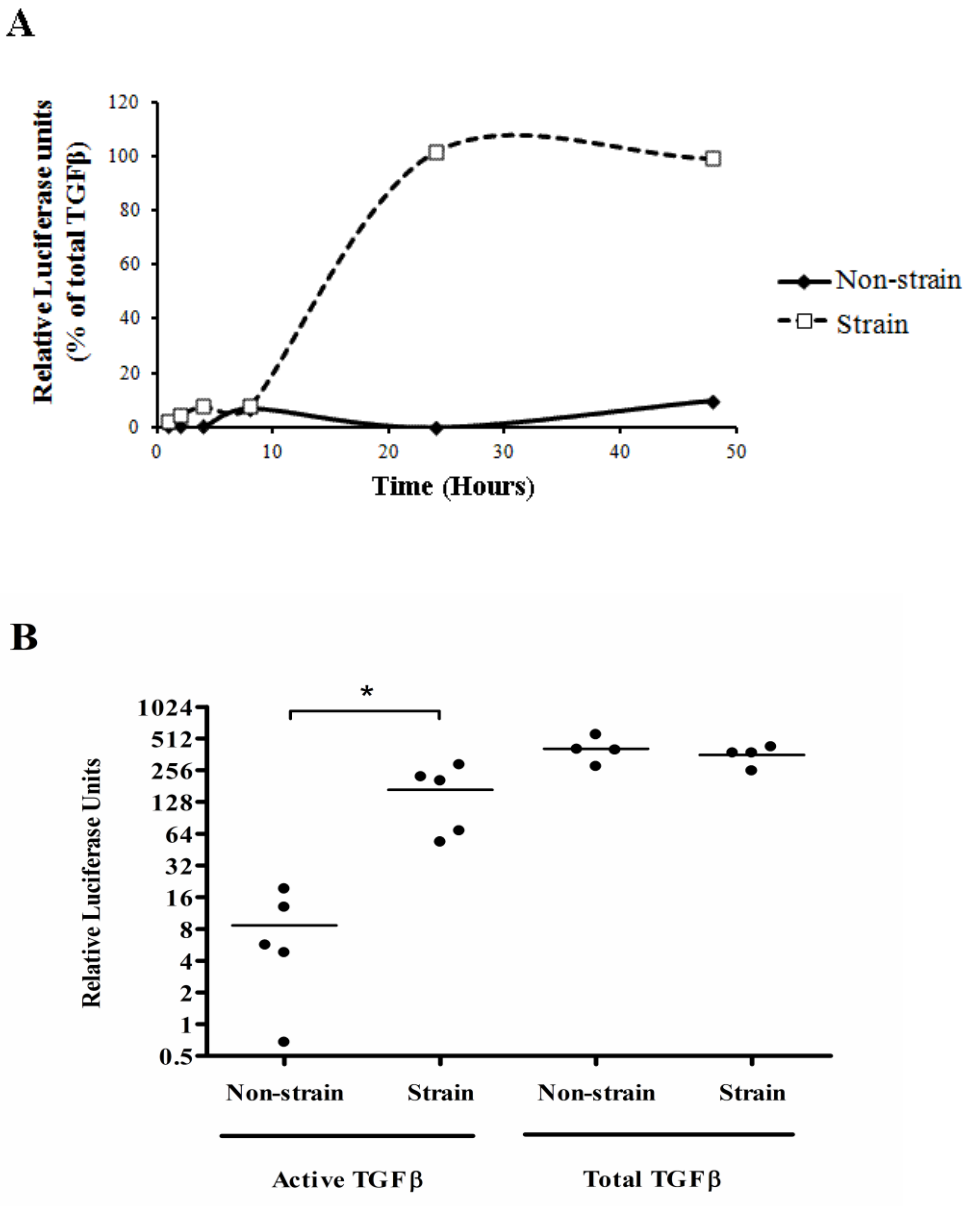

Figure 5.12. TGFβ Activation is increased with mechanical strain. A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see luciferase assay in methods section for details). Data is normalised to transfection controls and then negative controls before expression as a percentage of Total TGFβ (non-strain). In preliminary experiments TGFβ was measured over a time course period of 2, 4, 8, 12, 24 and 48 hours (A) (n=1). TGFβ was measured (total TGFβ from heat activated conditioned media cultures, active from untreated conditioned media) at 48 hours (B) (mean, n=5). Significant values are indicated as * according to the Wilcoxon signed rank test ($p<0.05$).

5.4. DISCUSSION

In this chapter we have shown that mechanical strain regulates multiple protease and matrix genes at the mRNA level and that changes in mRNA level are analogous to those induced by TGFβ stimulation. Furthermore, the inhibition of the TGFβ signalling pathway abrogated the strain-induced changes in mRNA level, demonstrating signalling via TGFβRI which mediates downstream phosphorylation of SMAD (Callahan, Burgess et al. 2002). This indicates that signalling is mediated via the canonical TGFβ signalling pathway which involves SMAD translocation to the nucleus and consequent transcriptional regulation. In further support of this we have shown that SMAD activation was similar in strained and non-strained samples in heat activated conditioned medium (total TGFβ) but significantly increased in strain conditioned medium (non heat activated, active TGFβ) compared to controls, suggesting that total TGFβ in strained and non-strained samples was similar, and that activation rather than synthesis of TGFβ is important in the mechanical regulation we have observed. We therefore hypothesise that application of 5% strain at 1Hz in our model induces TGFβ activation, subsequent signalling and regulation of genes in response to mechanical load.

We have observed that the vast majority of changes reported in response to strain have shown a similar pattern of response to TGFβ. Some of the gene changes in response to TGFβ have previously been reported in other cell types. For example TGFβ is reported to regulate ADAM12 (Le Pabic, Bonnier et al. 2003), ADAMTS1 (Cross, Chandrasekharan et al. 2005), ADAMTS4 (Yamanishi, Boyle et al. 2002; Corps, Jones et al. 2008), ADAMTS16 (Surridge, Rodgers et al. 2009), TIMP3 (Su, DiBattista et al. 1998), TIMP4 (Hyc, Osiecka-Iwan et al. 2011), Thrombospondin (Nakagawa, Li et al. 2004), aggrecan (Watanabe, de Caestecker et al. 2001), tenascin-C (Pearson, Pearson et al. 1988), collagen and fibronectin (Ignotz and Massague 1986). These responses are analogous to those seen in our model with TGFβ and strain, further supporting our conclusion that TGFβ plays a key role in the strain regulation of most metalloproteinase genes. ADAMTS5 has been shown to either be unresponsive (Yamanishi, Boyle et al. 2002) or to decrease (Cross, Chandrasekharan et al. 2005) in response to TGFβ. This corresponds with the current study in that induction of ADAMTS5 by strain is not related to TGFβ signalling directly. Recent research has also reported that ADAMTS5 can also positively regulate TGFβ signalling

through degradation of aggrecan, allowing TGFβ to access receptor molecules (Velasco, Li et al. 2011). The increase of ADAMTS5 in our system in response to strain may therefore increase TGFβ signalling, if this is reflected in ADAMTS5 expression at the protein level. Although MMP13 shows similar response to strain and TGFβ, regulation does not appear to be mediated via the TGFβRI pathway either. This indicates that regulation of MMP13 by TGFβ is via another TGFβ signalling pathway.

As previously described in the introduction, TGFβ protein is increased in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002). Pathway analysis following global gene expression profiling of chronic tendinopathic tendon tissue also showed an increase in genes related to TGFβ signalling (Jelinsky, Rodeo et al. 2011). In the previous chapter we have compared gene regulation in tendinopathy and response to mechanical load. We showed that there were many similarities. However expression also suggested that the mechanotransduction mechanism has been disrupted in some way in tendinopathy as there were also some differences in the response to mechanical load and those seen in tendinopathy. This indicates that TGFβ signalling response to load may be disrupted in some way. Abnormal loading regimes may result in differential response of TGFβ and contribute to tendinopathy development.

The majority of gene changes in response to strain and TGFβ appear to be very similar in most cases. However in some instances there is an additive effect when both strain and TGFβ are applied. ADAMTS5 is increased significantly with strain after 24 hours (2.2 fold), in response to TGFβ there was a trend to increase, however with a combination of strain and TGFβ treatment ADAMTS5 was increased further than strain alone (2.8 fold, 24 hours). Fibulin-1 was decreased with strain at 48 hours (2.3 fold) with TGFβ (3 fold) and was decreased further with a combination of the two conditions (4.9 fold). At 8 hours TGFβ2 was decreased 1.3 fold with TGFβ, remained unchanged with strain but was decreased further (2.8 fold) with a combination of TGFβ and strain. This indicates that either; strain and TGFβ responses in these cases rely on different mechanisms, i.e. the response is not TGFβ signal mediated, or the accumulation of strain activated TGFβ and the addition of active TGFβ may induce a more robust response. We have shown that fibulin-1 regulation with mechanical strain is reliant on the TGFβRI signalling, this suggests that in this case the latter explanation is more likely. Fibulin-1 therefore seems to be more sensitive to accumulation of TGFβ, different from many of the other genes that are responsive to strain via TGFβRI signalling. However, ADAMTS5 stimulation with strain

is not mediated via TGFβRI signalling therefore the former could be true. We have not confirmed whether the regulation of TGFβ2 is mediated via TGFβRI signalling therefore we cannot speculate as to why this occurs. This indicates that for these different genes there may be different modes of regulation. Mechanotransduction and the resulting modification of metalloproteinase and matrix genes is therefore complex. To complicate things further we have noted that Elastin showed a decreased response when strain and TGFβ were combined; Elastin was increased 3.8 fold with strain, 27.5 fold with TGFβ and 11.2 fold with strain and TGFβ in combination. We have shown that Elastin was regulated via TGFβRI however we were unable to fully characterise this response. In speculation, this may result from a negative feedback mechanism triggered when the levels of TGFβ reach a threshold; more research is required to test this hypothesis.

Even in the non-strained control cultures the addition of TGFβRI inhibitor caused mRNA levels to change in the opposite direction to the strain response, indicating a basal level of TGFβ signalling in non-strained cultures. This may be due to the tensional forces present across the tenocyte seeded collagen gel in the absence of cyclic loading. Even though the statistical values are close to the cut off for significance it is clear by the consistency of response between genes that TGFβRI is involved in their mechanotransduction.We have shown that TGFβ isoforms are not significantly increased with mechanical loading. However TGFβRI is increased with strain. The response of TGFβRI to strain paired with the increased in active TGFβ would result in an increased level of TGFβ signalling and therefore sensitising the TGFβ signalling pathway to the increase in active TGFβ. However to confirm this we would need to also measure TGFβRI at the protein level, perhaps by immunolocalisation studies. It would also be interesting to look at other TGFβ receptors such as TGFβRII and TGFβRIII which are also involved in the regulation of TGFβ signalling, however TGFβRI is the primary receptor involved in the canonical TGFβ signalling pathway.

Our results have shown that in many cases the response to TGFβ tends to occur earlier than the response to mechanical loading: for example all the ADAMTSs, TIMP3, Fibronectin, thrombospondin-1, Tenascin C, Scleraxis, IL6, IGF1, CTGF, Elastin and TGFβRI all respond to TGFβ much quicker than they respond to strain alone. As the majority of these genes are being regulated by TGFβ signalling in response to mechanical load this is reflected in the temporal delay of gene regulation. We have shown that TGFβ is activated between 12 and 24 hours, at which point we see regulation of strain responsive genes. With

TGFβ the response is seen as early as 2 hours. Genes that show a later response to TGFβ, such as COL1A1, may be regulated by TGFβ indirectly because of the delay in response to TGFβ treatment. This could be mediated by CTGF, as CTGF has been shown to act as a downstream mediator of collagen synthesis (Duncan, Frazier et al. 1999). Pathway analysis is required to confirm this mechanism.

COX2 and IL6 are regulated as early as 2 hours with mechanical strain. This suggests that they are responsive to mechanical loading by a mechanism that does not involve the activation of TGFβ at such as early time point. We have shown that IL6 response to mechanical loading at 24 and 48 hours involves TGFβRI signalling as inhibition of TGFβRI phosphorylation of SMAD proteins abrogates this strain induced response (data not shown). However we have not tested the expression of COX2 with the use of this same inhibitor, therefore we cannot confirm the involvement of TGFβ in the regulation of COX2 after 24 hours. However it seems likely that the early response of these genes to load does not involve TGFβ activation as activation only occurs after 12 hours. COX2 or IL6 may be involved in the activation of TGFβ via some other signalling pathway.

Taken together our data suggest that activation of TGFβ and subsequent TGFβ signalling is part of the mechanotransduction response of tenocytes to moderately high levels of strain. TGFβ up-regulation at both the protein and mRNA level with mechanical strain has been previously reported in tenocytes (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004) and other cell types (Riser, Cortes et al. 1996; Riser, Ladson-Wofford et al. 1999; Kim, Akaike et al. 2002; Baker, Ettenson et al. 2008). TGFβ has also been implicated as a regulatory step in the mechanical regulation of collagen (Yang, Crawford et al. 2004; Heinemeier, Olesen et al. 2007), although these studies have not specifically shown that activation of TGFβ is involved. Maeda and colleagues (Maeda, Sakabe et al. 2011) have reported TGFβ activation as a key regulator in scleraxis (a transcription factor expressed in tendon). However, we are the first to implicate TGFβ activation as a key regulator in the mechanoregulation of metalloproteinases and other matrix genes. As ADAMTS5 is not regulated via the same TGFβ signalling pathway, this suggests there is an alternative mechanotransduction pathway. This pathway may involve crosstalk with or may precede TGFβ activation. The next goal is to elucidate the mechanism by which mechanical strain induces latent TGFβ activation. As doing so could help us to fully understand how we could treat and ultimately prevent tendon disease.

5.5. CONCLUSIONS

1. The majority of matrix gene and metalloproteinase respond to strain in are very similar manner to TGFβ.
2. The majority of strain regulated changes of metalloproteinase and matrix genes is facilitated via TGFβRI.
3. TGFβ isoforms are not significantly increased with mechanical load at the mRNA level. However, TGFβRI mRNA is significantly increased with mechanical load.
4. TGFβ activation and not total TGFβ is increased in response to mechanical load.
5. Two exceptions; ADAMTS5 and MMP13 may be regulated via another mechanotransduction mechanism.

CHAPTER 6: How is TGFβ activated in response to mechanical loading?

6.1. INTRODUCTION

We have shown in the previous chapter that TGFβ activation occurs in response to mechanical loading in primary Achilles tenocytes, and that activation of TGFβ leads to the regulation of metalloproteinases, TIMPs and matrix genes. TGFβ is regulated on a number of levels including intracellular processing and the activation of a latent complex; several mechanisms of TGFβ activation have been defined in non tendon tissues. There are three main channels of activation which include protease cleavage, activation through integrin mediated tensional forces and the interaction of thrombospondin with LAP, generating either diffusible or non-diffusible TGFβ.

Newly synthesised TGFβ protein contains a pro-peptide known as the latency associated peptide (LAP). In the trans-golgi network, post-translational modifications involve cleavage of LAP by the proprotein convertase furin (Dubois, Blanchette et al. 2001). TGFβ and LAP remain non-covalently bound rendering TGFβ inactive (Gentry and Nash 1990). However the process of inhibition is not yet fully understood. Latent TGFβ binding proteins (LTBP) form cysteine disulphide linkages with TGFβ homodimers via LAP, to form the Large Latent Complex (LLC) (Gleizes, Beavis et al. 1996; Saharinen, Taipale et al. 1996). LTBP is important in the secretion (Miyazono, Olofsson et al. 1991) and targeting of LLC to the extracellular matrix where transglutaminases covalently link the LTBP to ECM proteins such as fibronectin (Taipale, Saharinen et al. 1996; Nunes, Gleizes et al. 1997; Verderio, Gaudry et al. 1999).

In the ECM, the LLC is a convenient store of potentially active TGFβ. Protease mediated activation of TGFβ can involve the activity of various proteases such as MT1-MMP,

MMP2, MMP3, MMP13, plasmin and thrombin (Lyons, Keski-Oja et al. 1988; Taipale, Koli et al. 1992; Yu and Stamenkovic 2000; D'Angelo, Billings et al. 2001; Maeda, Dean et al. 2002; Mu, Cambier et al. 2002). They can activate TGFβ by cleaving LAP from the latent TGFβ complex thereby releasing active TGFβ into the extracellular space. Surface localisation of proteases can involve M6P/IGF-II, CD44 or integrin interaction via RGD motifs. This releases active TGFβ pericellularly (Dennis and Rifkin 1991; Yu and Stamenkovic 2000; Mu, Cambier et al. 2002; Asano, Ihn et al. 2005). Integrins are also involved in the activation of TGFβ in the absence of protease activity. This involves tensional forces between the LLC sequestered in the ECM and the cellular cytoskeleton through the interaction of integrins with LAP (Munger, Huang et al. 1999; Wipff, Rifkin et al. 2007). This mechanism does not involve release of diffusible TGFβ but involves a conformational change and activation of TGFβ. Thrombospondin can also activate TGFβ. This occurs through the interaction of the LSKL motif with LAP and results in a conformational change and consequent activation of TGFβ without the release from the LAP (Schultz-Cherry and Murphy-Ullrich 1993; Crawford, Stellmach et al. 1998; Ribeiro, Poczatek et al. 1999). TGFβ is therefore regulated on many levels, through intracellular post-translational modification, localisation to the ECM and through a variety of activation mechanisms (For more details of TGFβ activation mechanisms see introduction).

The aim of this experiment is to target known mechanisms of TGFβ activation to determine whether they are involved in the activation of TGFβ in response to mechanical loading. To do this a range of protease inhibitors to inhibit protease cleavage of TGFβ, an RGD inhibitory peptide preventing integrin binding to TGFβ, an inhibitory antibody against integrin β1, an LSKL peptide to prevent interaction of thrombospondin with LAP and a M6P peptide to inhibit M6P/IGF-II interaction with LAP were applied (see table 6.1 for a list of inhibitors and their functions).

6.2. METHODS

Inhibition of mechanisms of TGFβ activation: Tenocytes seeded (1.5 x 10^6 cells/ml) in type I collagen (1mg/ml) were plated into tissue train Flexcell™ plates and uniaxially strained at 5% at 1Hz for 48 hours with or without GM6001 and its inactive control, Pefabloc, Protease inhibitor cocktail, Pepstatin, Hirudin, RDG peptide inhibitor and control peptide, MAB13 antibody against integrin β1, LSKL mimetic peptides and an unspecific control (SLLK) and Mannose-6-Phosphate peptide inhibitor (for details of concentrations and functions see table 6.1). We were unable to complete dose response experiments for all these inhibitors due to constraints of time and funding, concentrations used were chosen on the basis of those used in previous experiments (see references in table 6.1). Medium was collected for analysis in the TGFβ Luciferase assay and gels were dissolved in Trizol for further RNA analysis. For further details of the Flexcell™ setup see methods section 2.2.2.

RNA analysis: RNA was extracted using the tri-spin method. RNA was quantitated, reverse transcribed and analysed using qRT-PCR for a selection of metalloproteinase and matrix genes. Data was normalised to TOP1 and expressed as the $2^{\Delta Ct}$ (mean ± SE or SD) Statistical analysis was performed using either the students t-test (two way, paired) or the Wilcoxon signed rank test depending on the number of repeats and normal distribution. For full details of protocols see methods section 2.3.

TGFβ Luciferase assay: TGFβ was measured using a cell based system targeting SMAD proteins involved in TGFβ signalling. Medium from 48 hour strained gels was transferred onto SW1353 cells transfected with a SMAD specific construct linked to a luciferase reporter. Total TGFβ was measured by heat activating the TGFβ in the medium before incubation with the SW1353 cells. Luciferin was added to samples and fluorescence measured on the luminometer. For details of this protocol see general methods section 2.4.1.

Inhibitor	Concentration	Function
GM6001	10μM	Broad spectrum inhibitor of metalloproteinase activity (Li, Chen et al., 2008)
Pefabloc	0.5mM	An inhibitor of Serine proteases (Nunes, et al. 1995)
Protease inhibitor cocktail	1/500 dilution	contains 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin. PIC inhibits a variety of proteases with broad specificity; more specifically the inhibition of serine, cysteine, aspartic proteases and aminopeptidases.
Pepstatin	1μM	An Aspartate protease inhibitor
Hirudin	2U/ml	An inhibitor of Thombin (Lockwood, toti et al., 2005; Norwitz, Snegovskikh et al., 2007)
GRGDSP and control peptide GRGESP	13μM	RDG peptide inhibitor (Peptide sequence expressed by some integrins, that can bind LAP) (Munger et al., 1998; Choi, Kim et al., 2010)
MAB13	20μg/ml	antibody against integrin β1 Given to us by Martin Humpheries
LSKL mimetic peptides and SLLK unspecific control	13μM	LSKL is a peptide sequence within Thrombospondin that binds to LAP. (Kondou et al., 2003; Meek et al., 2003)
Mannose-6-Phosphate	10μM	A peptide that inhibits the interaction between M6P/IGF-II and LAP (Nunes et al., 1995)

Table 6.1. Summary of Inhibitors and their functions. References listed in the right hand column are those used to determine concentration for in the current study (Choi, Kim et al. 2010) (Nunes, Shapiro et al. 1995; Munger, Harpel et al. 1998; Kondou, Mushiake et al. 2003; Meek, Cooney et al. 2003; Lockwood, Toti et al. 2005; Norwitz, Snegovskikh et al. 2007; Li, Chen et al. 2008). Where no reference is listed the concentration suggested by the supplier was used.

6.3. RESULTS

6.3.1. Protease inhibition studies

There was no significant difference in the response to mechanical load in the level of active or total TGFβ in the presence of GM6001 compared to non-treated controls (Figure 6.1 A). RNA analysis revealed no significant difference in MMP1, MMP13, ADAMTS5 or Elastin response to mechanical loading in the presence of GM6001 (Figure 6.1 B). We have confirmed that GM6001 was active even after 48 hours in culture (see optimisation section 3.4).

The response of active TGFβ to mechanical load was not significantly changed with the addition of pefabloc compared to non-treated controls (Figure 6.2 A). There was no significant change in Total or active TGFβ. MMP1 mRNA was decreased in both strain and non-strain conditions in response to pefabloc treatment (Figure 6.2 B).

Neither active nor total TGFβ regulation with strain was clearly affected by the addition of protease inhibitor cocktail (PIC) (Figure 6.3 A). MMP1 and MMP13 mRNA regulation with mechanical load was unaffected by the addition of PIC (Figure 6.3 B). However in ADAMTS5 there was a reduction in the response to strain with the addition of PIC. Elastin failed to show a clear response to mechanical load in this particular cell isolate.

Pepstatin did not substantially alter the regulation of active or total TGFβ with strain (Fig 6.4 A). There was a trend to decrease in the level of active TGFβ in the presence of pepstatin. MMP1, MMP13, ADAMTS5 and Elastin actually showed an increase in the response to mechanical loading compared to non-treated controls (Figure 6.4 B).

Responses to mechanical loading were not substantially altered with the addition of hirudin; however there was a trend to increase in active and total TGFβ with the addition of hirudin in both strained and non-strained cultures (Figure 6.5 A). A similar response to mechanical loading was seen in cultures with or without hirudin in MMP1, MMP13 and ADAMTS5 (Figure 6.5 B). However Hirudin did cause a decrease in the strain response in Elastin.

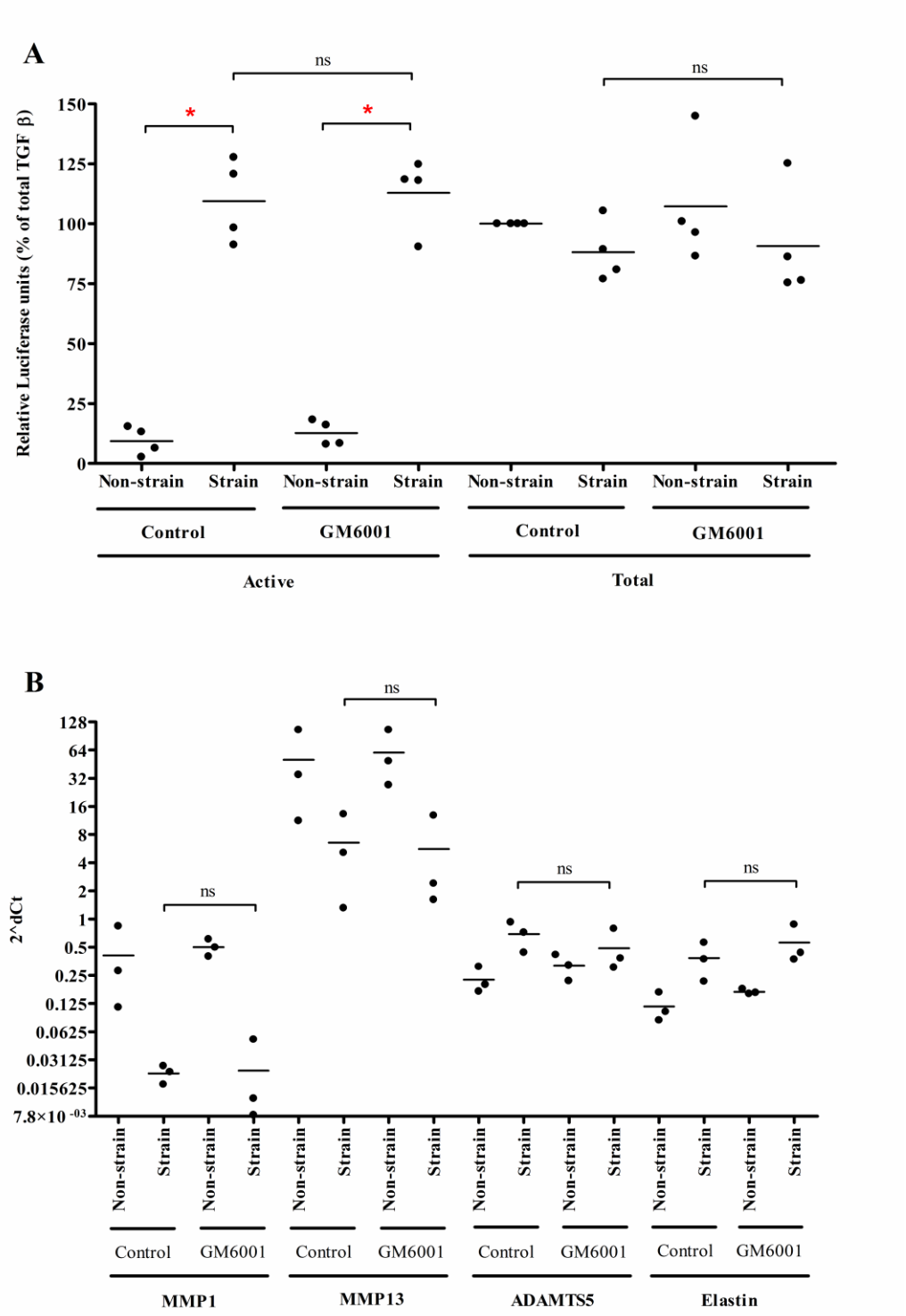

Figure 6.1. GM6001 has no effect upon strain regulated TGFβ activation or strain regulated gene expression. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). GM6001 and its inactive control were added before the straining protocol (10µM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean, n=3). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the mean fold change with strain (n=3). Significant values are indicated as * ($p<0.05$) and ns = not significant; according to the t-test (2 way, paired).

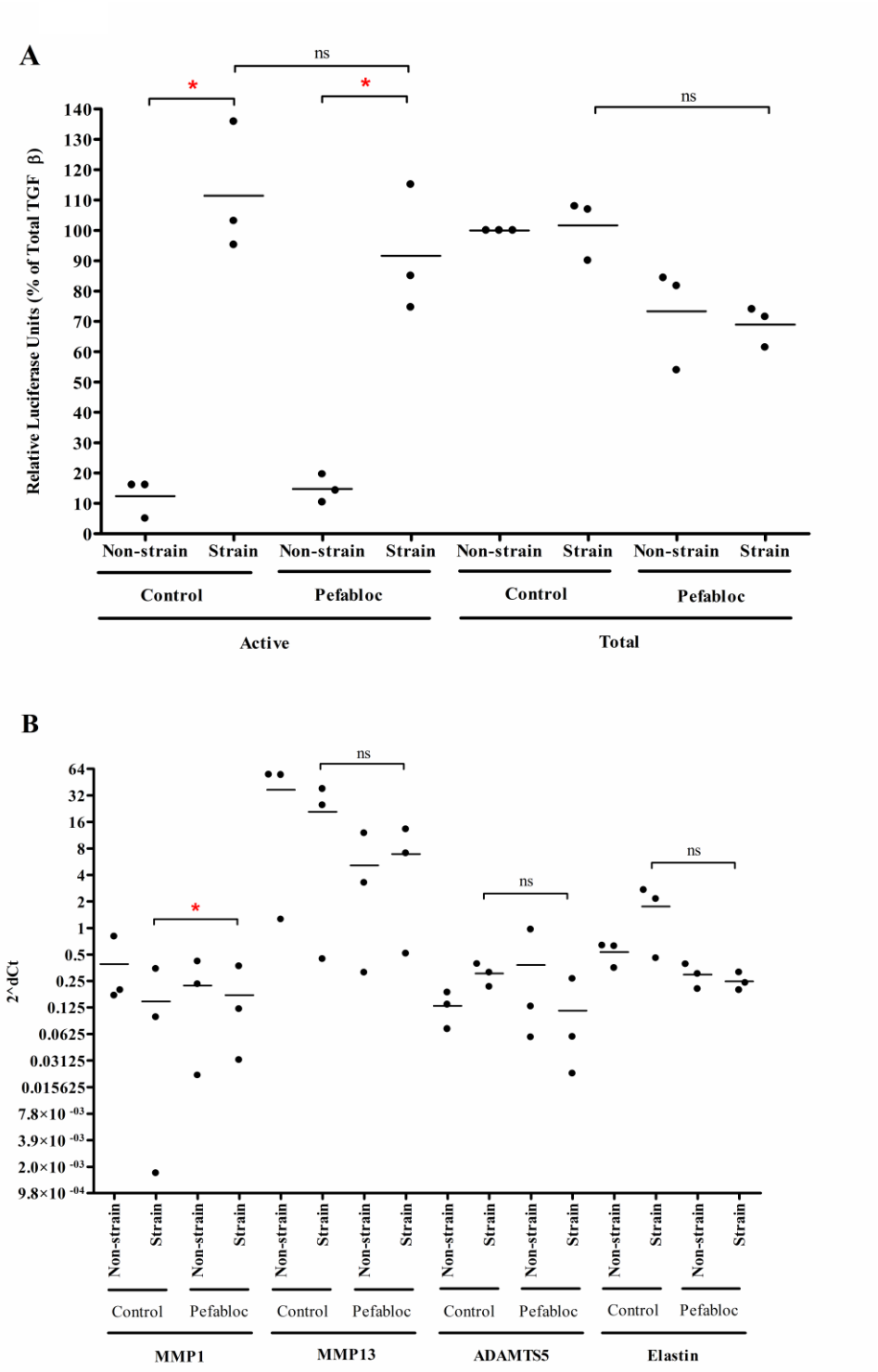

Figure 6.2. Pefabloc has a small effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Pefabloc was added before the straining protocol (0.5mM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean, n=3). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as $2^{\Delta Ct}$, to highlight the differential regulation in both strain and non-strained samples (mean, n=3). Significant values are indicated as * ($p<0.05$) and ns = not significant; according to the t-test (2 way, paired).

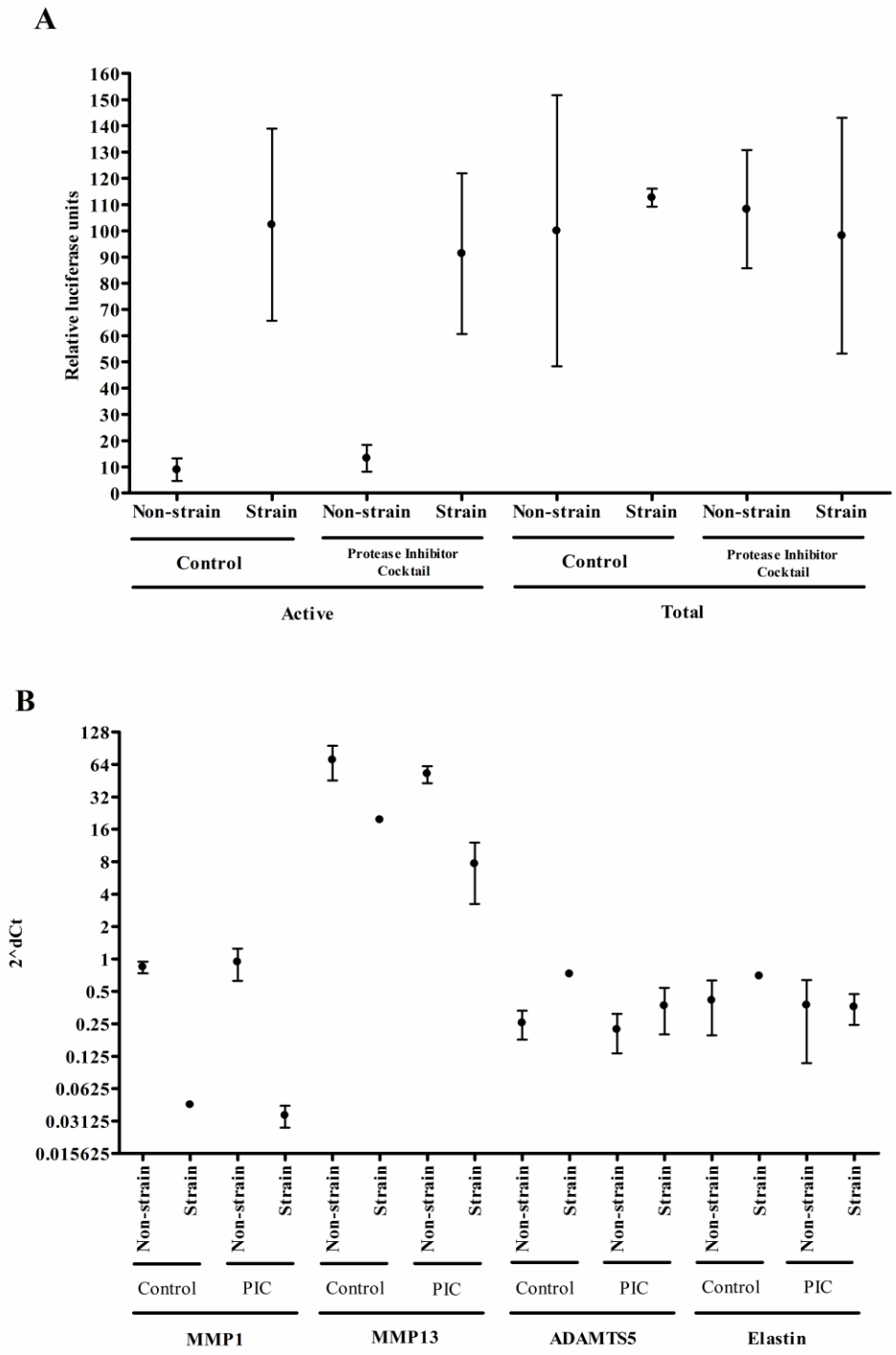

Figure 6.3. Protease inhibitor cocktail has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Protease inhibitor cocktail was added before the straining protocol (1/500 dilution). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean ±SD, n=1). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as $2^{\Delta Ct}$, to highlight the differential regulation in both strain and non-strained samples (mean ±SD, n=1).

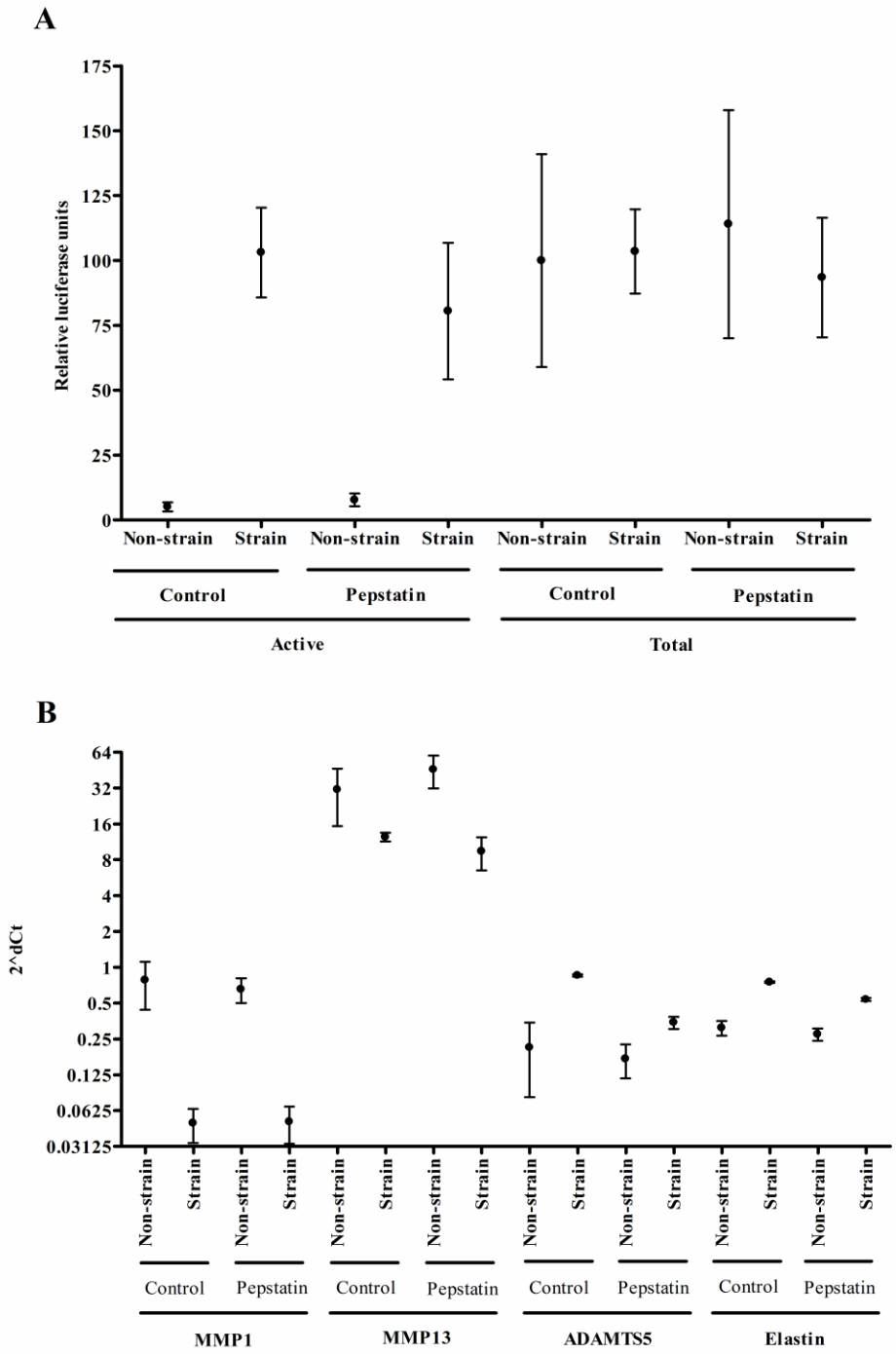

Figure 6.4. Pepstatin has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Pepstatin was added before the straining protocol (1µM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean ±SD, n=1). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as $2^{\Delta Ct}$, to highlight the differential regulation in both strain and non-strained samples (mean ±SD, n=1).

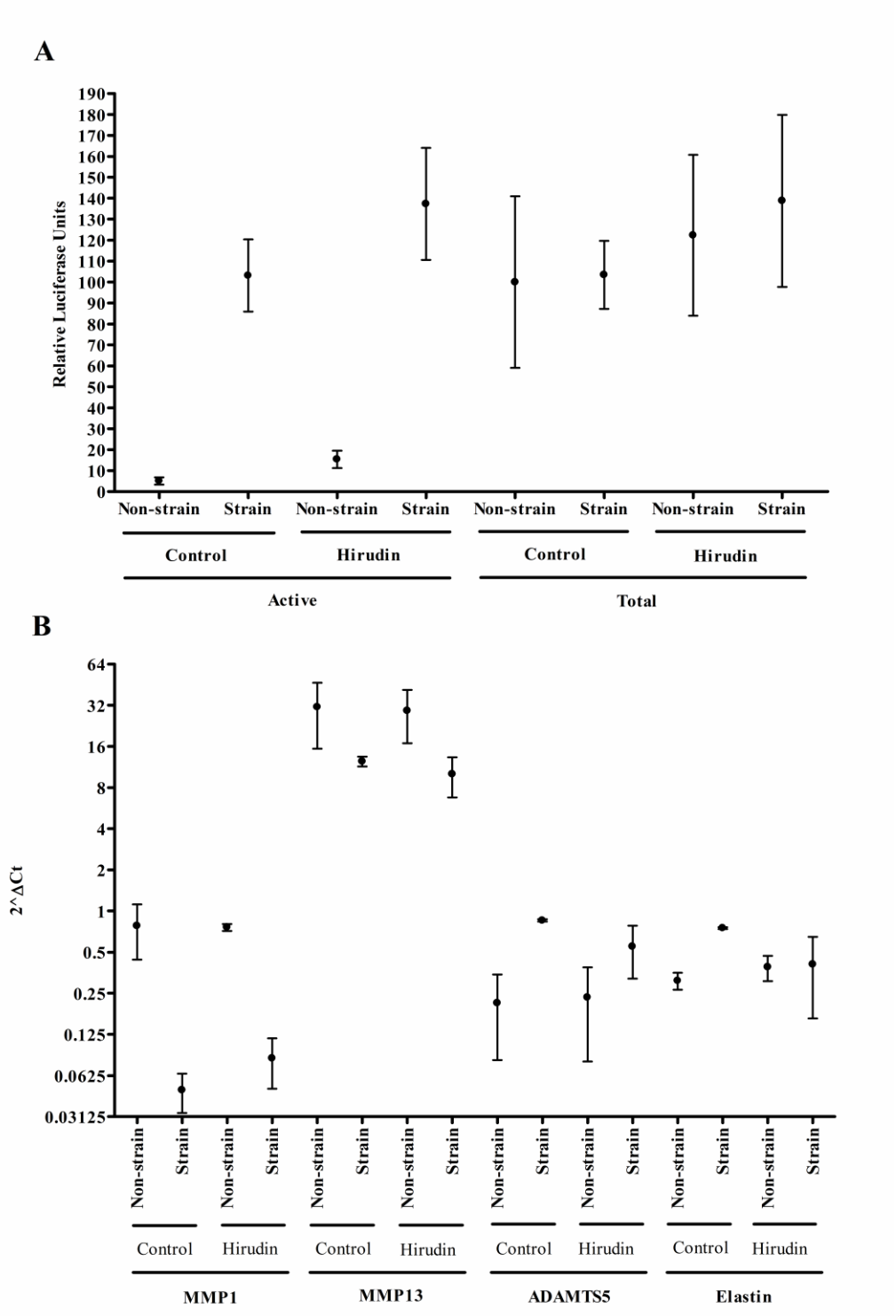

Figure 6.5. Hirudin has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Hirudin was added before the straining protocol (2U/ml). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean ±SD, n=1). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as a ratio of strain: non-strain (mean ±SD, n=1).

6.3.2. Inhibition of LAP-thrombospondin interaction

LSKL is an inhibitory peptide of the interaction between thrombospondin and LAP; SLLK is a control peptide similar in structure to LSKL but without the ability to inhibit LAP interaction. Mechanical loading induced an increase in the level of active TGFβ in control, SLLK and LSKL treated cultures. There was no significant change in strain induced active or total TGFβ with the addition of LSKL (Figure 6.6 A). MMP1 mRNA regulation did not show such a marked change with strain compared to previous experiments. MMP1, MMP13, ADAMTS5 and Elastin mRNA regulation with mechanical strain was not significantly different when LSKL was added (Figure 6.6 B).

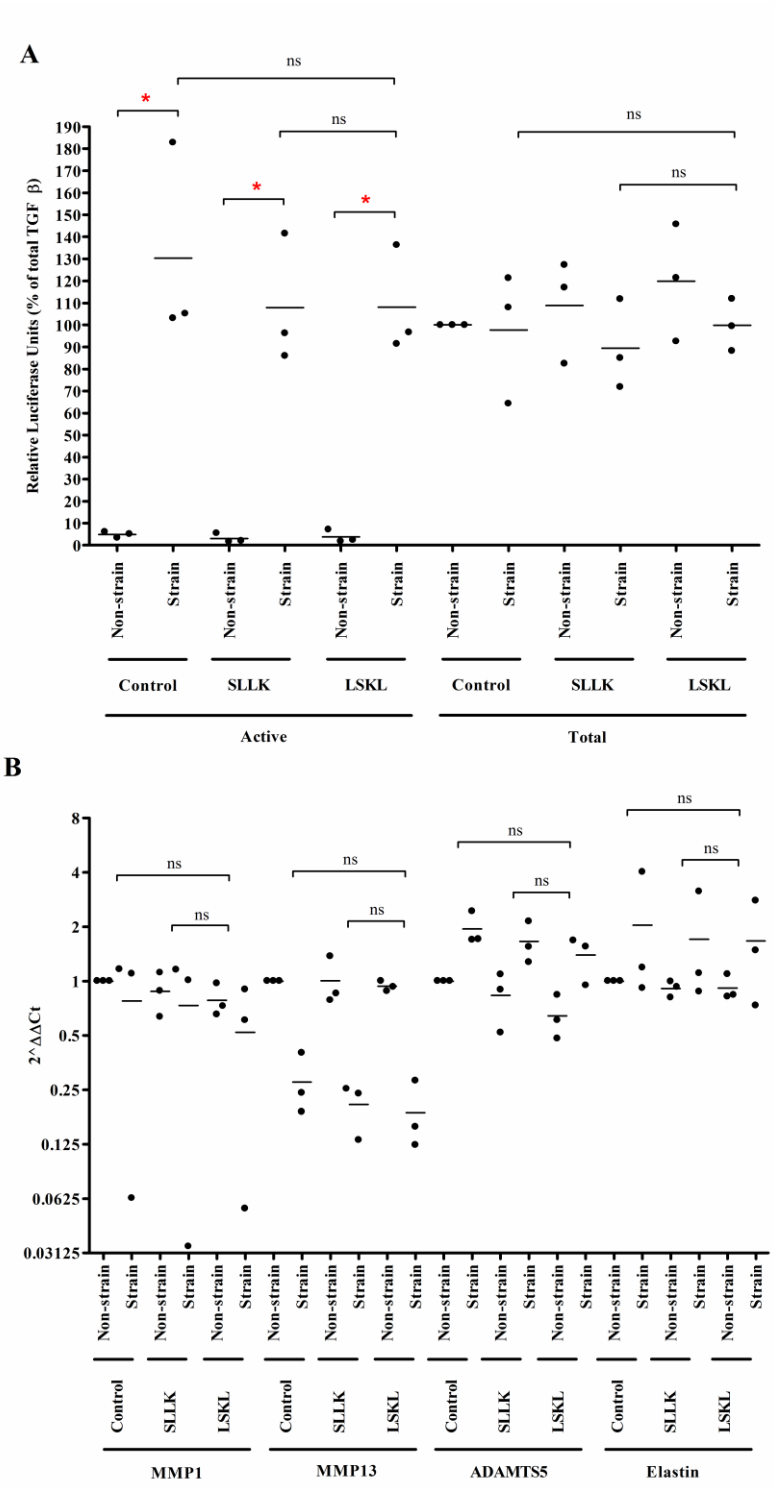

Figure 6.6. LSKL mimetic peptide has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). LSKL mimetic peptides and an unspecific control (SLLK) were added before the straining protocol (13μM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=3). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the mean fold change with strain (n=3). Significant values are indicated as * according to the t-test (2 way, paired) ($p<0.05$).

6.3.3. Integrin inhibition

Control and MAB13 (integrin β1 antibody) treated cultures showed a significant increase in active TGFβ in response to mechanical loading. However those treated with control IgG did not show a significant change (Figure 6.7 A). Total TGFβ was not significantly different between control and MAB13 treated cultures. MMP1 mRNA did not show the usual decrease in response to strain (Figure 6.7 B). Control IgG treatment showed a similar response to controls with mechanical loading. Treatment with MAB13 showed a significant increase in Elastin in response to mechanical loading.

There was no significant difference between control cultures and those treated with GRGDSP in terms of active TGFβ (Figure 6.8 A). Statistical analysis was only possible between strain and non-strain samples in control cultures and also between control and GRGDSP in strained cultures as the first repeat of this experiment did not include all conditions; restricting the statistical analysis to those conditions where three repeats were available. Again with the mRNA analysis we were restricted with statistical testing to those experiments that were of three repeats or more, however we were able to confirm that MMP1 and MMP13 regulation with strain were not significantly different in GRGDSP cultures compared to controls (Figure 6.8 B). ADAMTS5 and Elastin has similar patterns of regulation with strain in control, GRGESP and GRGDSP treated cultures, although this was restricted to n=2.

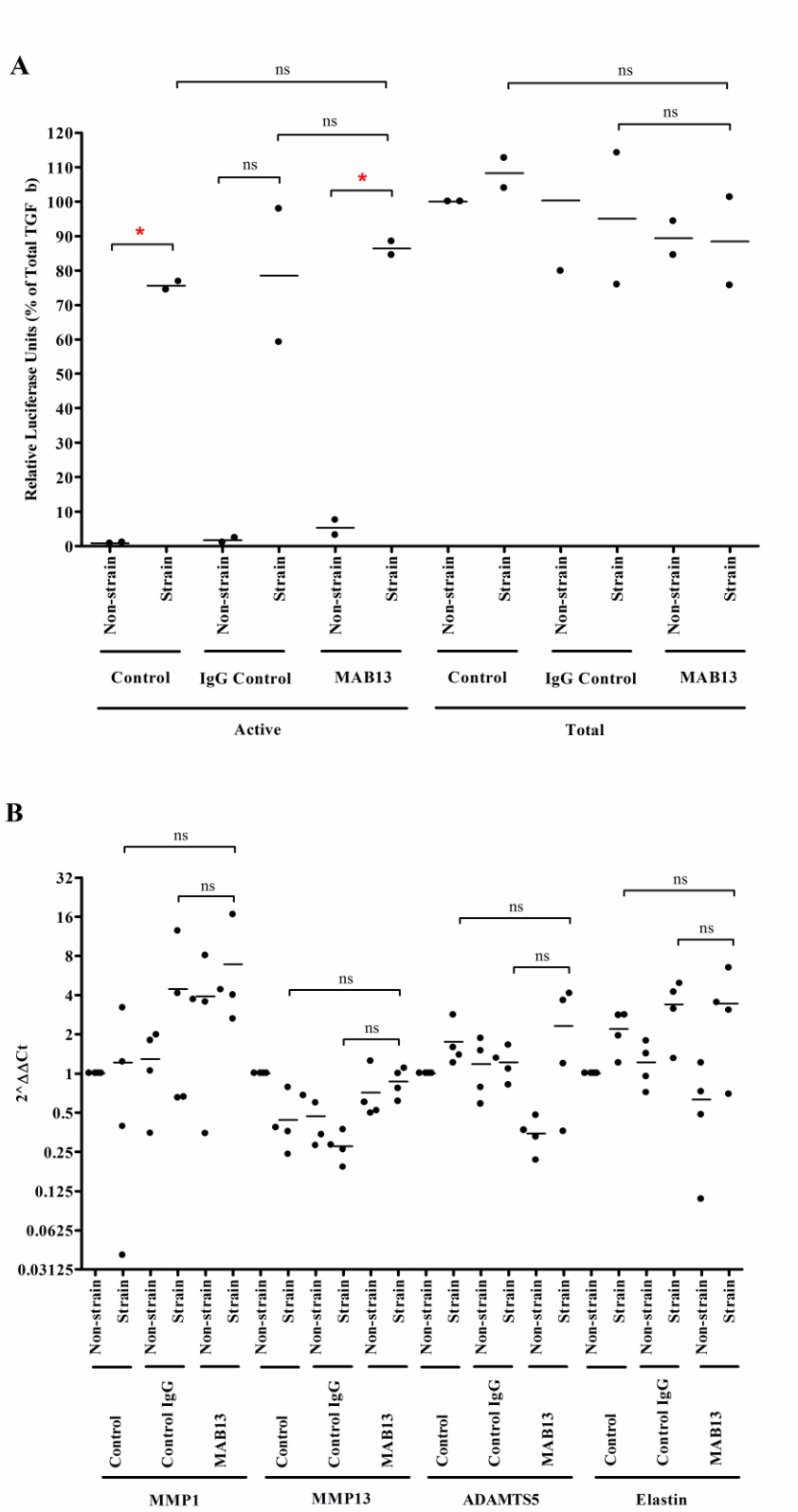

Figure 6.7. MAB13 β1 integrin antibody has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). MAB13 was added 30 minutes before the straining protocol (20µg/ml). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean, n=3). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the mean fold change with strain (n=4). Significant values are indicated as * according to the t-test (2 way, paired) ($p<0.05$).

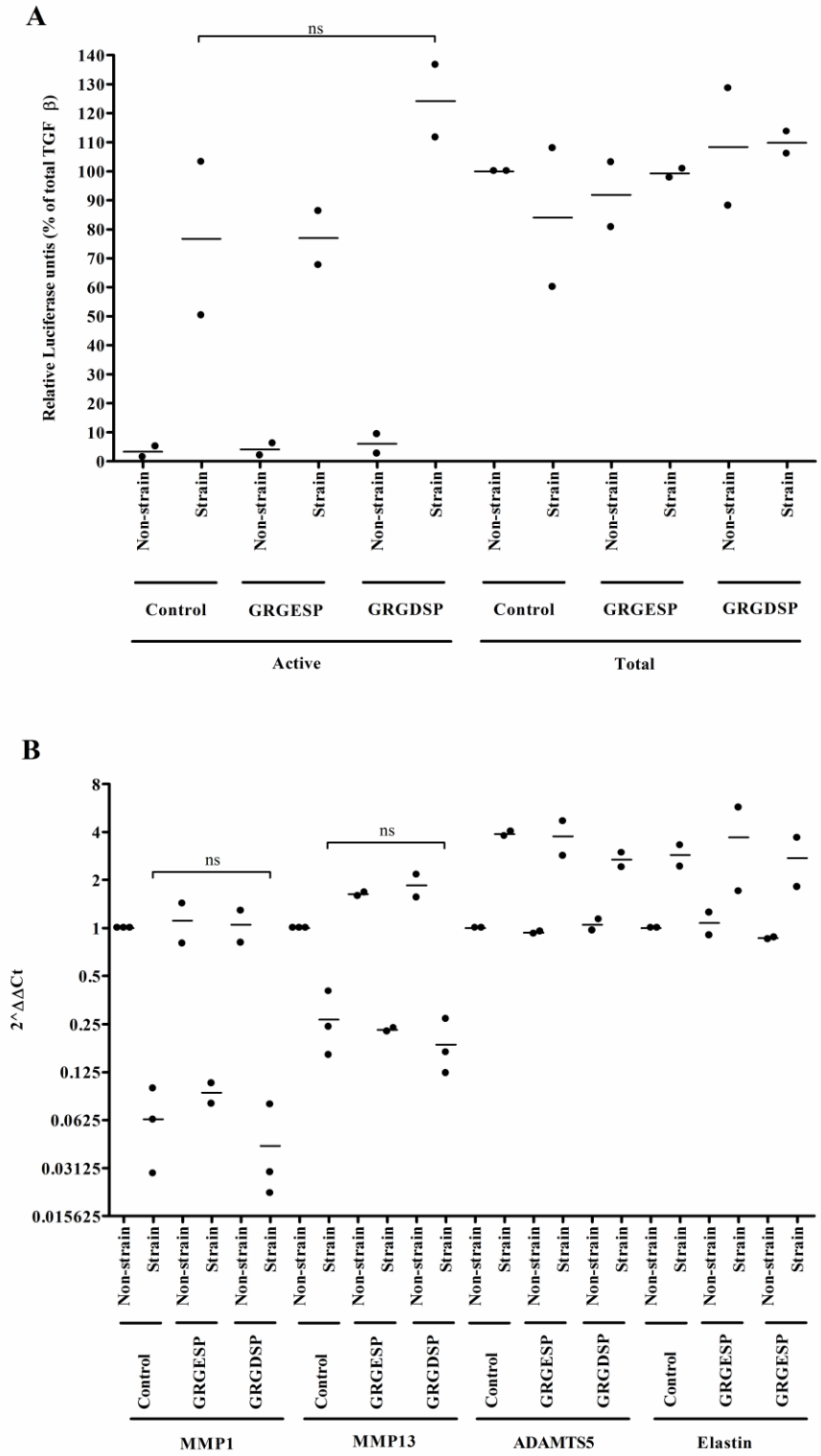

Figure 6.8. RGD peptide inhibition has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). GRGDSP and GRGESP were added before the straining protocol (7μM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (mean, n=3). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the mean fold change with strain (n=3). Significant values are indicated as *, according to the t-test (2 way, paired) ($p<0.05$).

6.3.4. Inhibition of TGFβ - M6P/IGF-II interactions

Although we were only able to repeat the luciferase assay to measure active and total TGFβ twice, we can see that the addition of M6P does not sufficiently affect the level of active TGFβ (Figure 6.9 A). There was no significant difference between control and M6P treatments in the presence of strain in MMP1, MMP13 or ADAMTS5 (Figure 6.9 B). However M6P significantly decreased Elastin mRNA expression compared to untreated controls in strained cultures, although this change was small (1.1 fold).

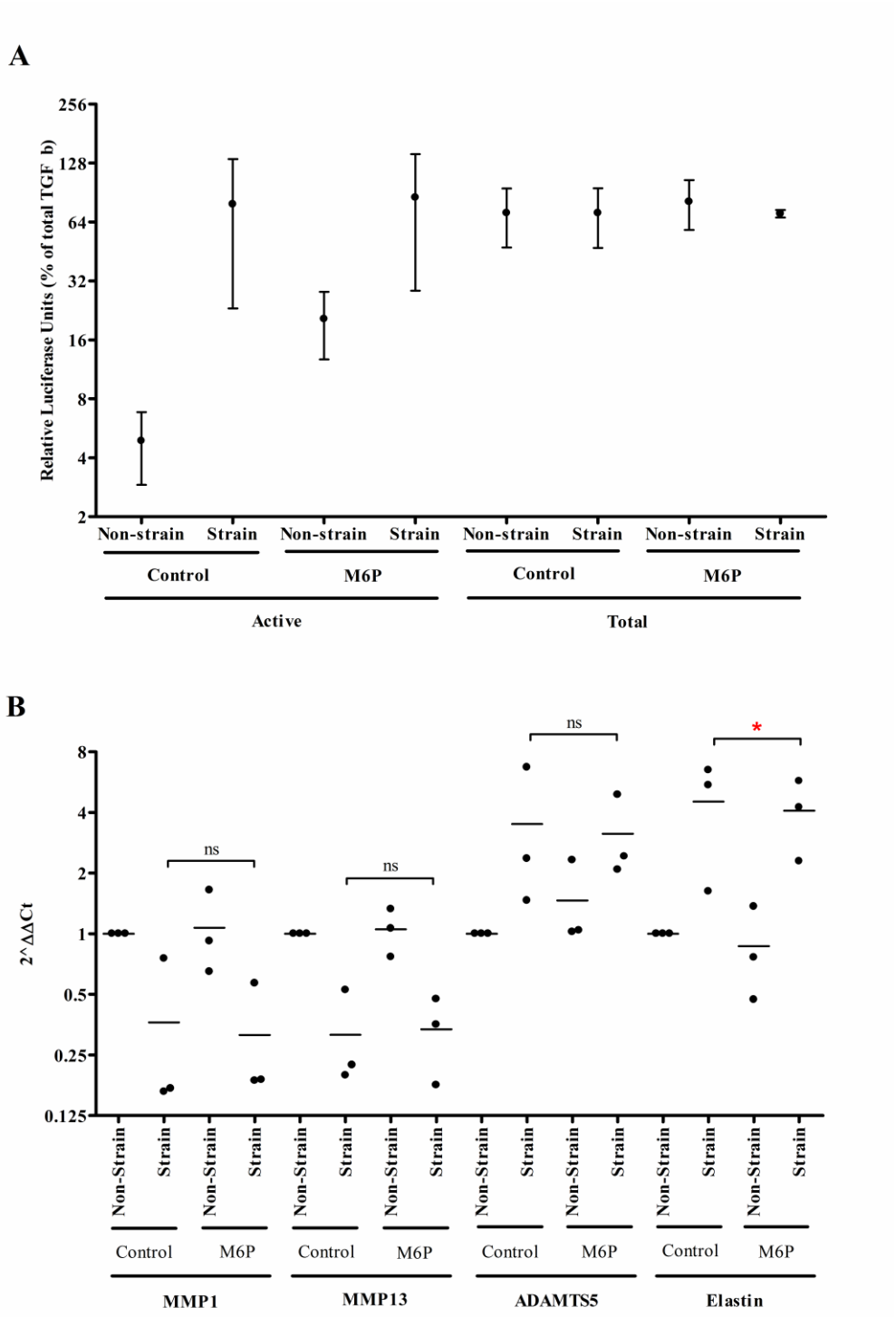

Figure 6.9. Mannose-6-Phosphate has no effect upon strain regulated TGFβ activation. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Mannose-6-Phosphate was added to cultures before the straining protocol (10μM). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=2, n=1 is shown this is representative of both repeats, mean ±SD). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the mean fold change with strain (n=3). Significant values are indicated as *, according to the t-test (2 way, paired) ($p<0.05$).

6.4. DISCUSSION

In this chapter we have shown that metallo, serine, cysteine, aspartic proteases and aminopeptidases are probably not involved in the activation of TGFβ in response to mechanical loading (through the use of GM6001, pefabloc, protease inhibitor cocktail, pepstatin and hirudin), despite the evidence to show that protease can play an important role in the activation of TGFβ in other systems (Lyons, Keski-Oja et al. 1988; Taipale, Koli et al. 1992; Yu and Stamenkovic 2000; D'Angelo, Billings et al. 2001; Maeda, Dean et al. 2002; Mu, Cambier et al. 2002). TSP1 has also been reported to be one of the key regulators of TGFβ activation in other systems (Schultz-Cherry and Murphy-Ullrich 1993; Crawford, Stellmach et al. 1998; Ribeiro, Poczatek et al. 1999). In addition there is evidence to suggest that shear force is able to stimulate the activation of TGFβ in blood platelets (Ahamed, Burg et al. 2008), and that activation of TGFβ is partially facilitated by TSP1 (Ahamed, Janczak et al. 2009). However, here we show that the interaction between thrombospondin and LAP via the LSKL motif is not involved in the activation of TGFβ in response to mechanical load; this could be due to differences in force (shear and tensional force) or cell type (blood platelets and tenocytes). M6P/IGF-II interaction with LAP has also been shown to regulate TGFβ activation in other tissues (Dennis and Rifkin 1991). Here we show that TGFβ activation does not involve the interaction of TGFβ and M6P/IGF-II via M6P.

Integrins have been reported as major regulators in the activation of TGFβ mainly via the RGD motif expressed by a subset of integrins (Yang, Mu et al. 2007; Aluwihare, Mu et al. 2009). We have shown that RGD interactions may be obsolete in the regulation of TGFβ activation in response to mechanical load in tendon cells. In addition β1 integrin may not play a role in this process of activation. We therefore propose that TGFβ activation in response to mechanical loading in tenocytes is mediated by an unknown mechanism.

As we have described in the introduction, TGFβ is targeted to the ECM in a large latent complex made up of the inactive TGFβ and the LLC. LTBP targets the LLC to the ECM where transglutaminase can bind the complex to components of the matrix such as fibronectin. The ECM is thought to act as a store of potentially active TGFβ. Therefore we should not be able to measure total TGFβ in the medium as usually this store of inactive TGFβ is sequestered in the ECM. In our system we have shown that both active and inactive TGFβ are detected in the medium. This suggests that, perhaps due to the lack of a more complex ECM (i.e. our cells are seeded into type I rat tail collagen),

transglutaminases are unable to sequester TGFβ LLC into the matrix and therefore TGFβ is detectable in the medium. It is likely that other ECM components such as fibronectin are laid down into the ECM as the cells are allowed to settle into the matrix for 72 hours before the loading regime is started. However an inability to attach to the matrix may be due to the lack of specific components such as mature fibronectin. Therefore the mechanism of activation in this simplified system may involve the activation of TGFβ as it is roams freely in the medium, which may rule out mechanisms of activation that involve tensional forces between the cells and the ECM. To further characterise the process of TGFβ activation we need to determine whether LTBP associates with TGFβ and whether the complex is sequestered in the ECM at any point. To do this we could use western blotting, ELISA or immune-localisation using specific LTBP binding antibodies.

Another possibility is that TGFβ is sequestered in the ECM but proteases cleave the LLC so that a processed form of the LLC becomes diffusible and open to further activation. However because none of our protease inhibitors had any effect upon levels of active or total TGFβ, this does not appear to be the case. However, this cleavage may be mediated by a protease that is not abrogated by the inhibitors we have tested. For example, as we have discussed in the introduction BMP1 can produce a diffusible processed LLC (Ge and Greenspan 2006), though BMP1 has metalloprotease activity it is unclear whether BMP1 is inhibited by any of the protease inhibitors we have used. Therefore specific inhibition of BMP1 could determine whether it is involved in LLC release from the ECM. In addition we would need to confirm the activation mechanism that follows this release, as there is no evidence that BMP1 can also achieve this.

MMP1, MMP13, Elastin and ADAMTS5 were chosen for RNA analysis in these inhibitor experiments to determine whether the inhibition of TGFβ activation mechanisms had any effect upon the regulation of these genes with mechanical strain. MMP1 and Elastin were chosen due to their robust responses and differential effects in response to mechanical loading. MMP13 and ADAMST5 were chosen because their regulation was independent of TGFβRI signalling; consequently if the inhibitors of TGFβ activation could inhibit MMP13 or ADAMTS5 regulation with strain we would be able to determine that another TGFβ pathway independent of TGFβRI was involved in their regulation. RNA analysis showed little difference in MMP1, MMP13, elastin and ADAMTS5 regulation with strain with the addition of the majority of these inhibitors. However, in cultures treated with pefabloc, MMP1 showed a trend to decrease in non-strained controls (not significant) and

an increase in strained cultures (highly significant, p=0.00678). This suggests that rather than inhibiting the strain induced response there was a more systemic effect. Similar results were seen in ADAMTS5 and elastin although these changes were not significant. Pepstatin induced an amplification of the strain induced decrease in MMP1 expression. Although not significant, Hirudin reduced the level of elastin and ADAMTS5 in response to mechanical strain and similar effects were seen with the protease inhibitor cocktail. This suggests that inhibition of thrombin may have some other effect upon ADAMTS5 and elastin that does not affect the mechanical regulation of MMP1 or MMP13. However because these experiments only looked at one cell isolate this cannot be confirmed at this stage.

The response of elastin to mechanical loading was not as marked in these inhibitor experiments as previously reported. This may be due to the cell isolates used in these particular experiments. In addition these experiments were run for 48 hours without stopping the loading regime. Previous studies focused on a time course which meant stopping the strain multiple times during the regime in order to harvest different time points. The lack of pause in this regime may have resulted in elastin reaching its peak of stimulation at an earlier time point. This has been previously noted in experiments looking at only 24 and 48 hours, where they have only been paused once during the regime. The regulation at 24 hours was similar to that seen after 48 hours in time course experiments, with levels beginning to return to control levels after 48 hours (Data not shown). M6P significantly decreased the level of elastin stimulated in response to mechanical loading. However this was only a very small change, with a statistical value very close to the cut off for significance (p=0.0442). Therefore M6P is unlikely to have a regulatory role in the absence of any affect in TGFβ activation.

Although we have looked at a wide range of TGFβ inhibition mechanisms, the study does have some limitations; many of our experiments were repeated less than three times due to time constraints, therefore they were not repeated enough times allow statistical testing. In addition, we were unable to confirm the effectiveness of all the inhibitors we used. However, we were able to confirm that GM6001 was capable of inhibiting MMP activity even after 48 hours of culture with tenocytes using a fluorescent substrate (see section 3.4). In addition, although we did not see the effect of these inhibitors upon the activation of TGFβ, some of the genes we measured showed a modified trend with inhibitor treatment, despite these changes not shedding light on strain mediated changes in gene expression. For example PIC reduced strain regulation of ADAMTS5, pepstatin induced an increase in

the strain response in MMP1, MMP13, elastin and ADAMTS5, and finally hirudin showed a decrease in the strain response in elastin. In addition the integrin β1 inhibitory antibody (MAB13) abrogated gel contraction seen in response to mechanical load (data not shown). This indicates that these inhibitors were effective at inhibiting there specific targets but did not affect TGFβ activation. However, neither the RGD nor LSKL peptide inhibitors showed any significant changes in terms of TGFβ activity or gene expression. Due to time constraints we were unable to confirm the inhibitory ability of these peptides. Confirmation of the activity of each of these inhibitors is required.

We have shown that known mechanisms of TGFβ activation are probably not responsible for the activation of TGFβ in response to mechanical load; therefore this could represent a novel mechanism of activation. However, to characterise this mechanism, further research is needed. The literature reports that other signalling pathways, such as calcium and MAPK signalling (Wall and Banes 2005), are important in mechanotransduction. Perhaps targeting these pathways may determine how TGFβ is activated or to characterise the signalling mechanism proceeding TGFβ activation. Integrins are important communicators with the ECM, other integrin motifs such as the collagen binding motif GFOGER (Xu, Gurusiddappa et al. 2000; Zhang, Kapyla et al. 2003) could be involved in the regulation of TGFβ activation. Due to time constraints we were unable to test this hypothesis. However, inhibition of integrin binding of collagen via the GFOGER motif may reveal a novel mechanism of TGFβ activation.

6.5. CONCLUSIONS

1. None of the known mechanisms of TGFβ activation are responsible for TGFβ activation in response to mechanical strain in our system.
2. A novel mechanism of activation could be responsible for strain activation of TGFβ in the current study.

CHAPTER 7: Further characterisation of the mechanotransduction mechanism in human tenocytes

7.1. INTRODUCTION

ATP, as well as ADP and adenosine can act as activators of purinoceptors. Purinoceptors are involved in multiple physiological functions including inflammation, peripheral and central neuronal transmission and muscle contraction. Purinoceptors are categorised into two main groups, those which respond to ATP or its analogs (ADP, AMP and GTP) and those which are activated by adenosine these are termed P2 and P1 purinoceptors respectively. P2 purinoceptors are further categorised into groups P2X and P2Y; P2X are ligand gated ion channels which are selectively permeable to cations such as calcium, whereas P2Y are G-protein coupled receptors (Valera, Hussy et al. 1994; North 2002; Gever, Cockayne et al. 2006). A number purinoceptor signalling pathways have been implicated in the process of mechanotransduction. For example, ATP is released from the cell in response to mechanical loading (Graff, Lazarowski et al. 2000; Jones, Wall et al. 2005); it can signal via P2 purinoreceptors. Activation of P2X purinoceptors (ligand gated ion channels) can cause the influx of cations such as calcium (Gever, Cockayne et al. 2006). P2Y activation via ATP can act in either an autocrine or paracrine fashion. Upon ATP binding to G coupled purinoreceptors (P2Y), G proteins are activated via GTP binding, this results in the activation of phospholipase C which cleaves phosphotidylinositol 4,5- bisphosphate (PIP_2) into Inositol-3-phosphate (IP_3) and 1,2 dicylglycerol (GAG). Inositol 1,4,5-triphosphate (IP_3) translocates into the cytoplasm and can act upon calcium sensitive ion channels located on the endoplasmic reticulum (ER), this results in the release of calcium from intracellular stores. Calcium, IP_3 and ATP can enter the cell or be released to signal to other cells through GAP junction hemi-channels or calcium channels (Naruse, Sai et al. 1998; Naruse, Yamada et al. 1998; Stout, Costantin et

al. 2002; Wall and Banes 2005). Calcium transients have been reported in some studies and not others, therefore the role of calcium in tendon mechanotransduction is not fully understood (Hung, Allen et al. 1997; Elfervig, Francke et al. 2000; Franke, Banes et al. 2000; Elfervig 2001; Elfervig, Lotano et al. 2001; Archambault, Elfervig-Wall et al. 2002; Wall and Banes 2004; Wall and Banes 2005).

P2Y1 and P2Y2 purinoceptors are responsible for downstream ATP signalling events (increase in intracellular calcium) in rodent Achilles tendon (Fox, Jones et al. 2005; Jones, Yang et al. 2005). Studies of various other organs have also shown that P2X and P2Y purinoceptors are involved in the response to mechanical load; these include response to compressive force upon human cartilage (P2Y) (Millward-Sadler, Wright et al. 2004), fluid flow in osteoblasts (P2Y) (You, Jacobs et al. 2002), fluid flow endothelial cells (P2X4) (Yamamoto, Korenaga et al. 2000) and mechanical stimulation of smooth muscle cells (P1 and P2 receptors) (Hamada, Takuwa et al. 1998).

By blocking calcium channels, phospholipase C and intracellular storage of calcium we hoped to confirm whether calcium signalling is involved in the response to mechanical load. The following inhibitors were selected: α18-Glycerrhitinic acid and GAP27 are gap junction inhibitors, α18-glycerrhitinic acid is a general gap junction blocker and Gap27 is a selective gap junction blocker specific to Cx43 (a mimetic peptide derived from the Gap27 domain of connexin 43). Thapsigargin is a non-competitive inhibitor of calcium ATPase and results in the increase of cytosolic calcium and depletion of intracellular calcium stores. U73122, Nifedipine and Amiloride are an inhibitor of phospholipase C, an L-type calcium channel blocker and a t-type calcium channel blocker respectively. Apyrase contains 2 isoenzymes; ATPase and ADPase they are involved in the degradation of ATP and ADP ($ATP \rightarrow ADp + Pi \rightarrow AMP + 2Pi$).

The MAPK network is involved in multiple cell processes. Components of MAPK signalling are reported to be involved in TGFβ signalling (Moustakas and Heldin 2005) and mechanotransduction in terms of stress deprivation (Kawabata, Katsura et al. 2009). For instance p38 MAPK is stimulated in response to stress deprivation and p38, MKK1 and PI3K are involved in TGFβ signalling crosstalk (Moustakas and Heldin 2005; Kawabata, Katsura et al. 2009). By inhibiting a range of these kinases we can determine whether they are involved in the mechanotransduction and consequent regulation of metalloproteinase and matrix regulation with mechanical strain or determine whether

signalling via MAPK pre or proceeds the activation of TGFβ. Two inhibitors for each of the following pathways were chosen: p38 MAPK (SB203580, Doramapimod), MKK1 pathway (PD0325901, U0126) and PI3K (Wortmannin, PI-103). Each of these inhibitors acts in a different manner to its partner inhibitor, therefore when both inhibitors effectively inhibit either TGFβ activation or gene regulation we can confirm which pathway is involved.

Previous studies have looked at the functional interaction of TGFβ and IL6. In intestinal epithelial cells TGFβ has been shown to abrogate the phosphorylation of STAT1 and STAT3 in response to IL6 stimulation. This suggests that TGFβ is able to modify the IL6 signalling pathway (Walia, Wang et al. 2003). A study of airway smooth muscle cells has shown that TGFβ can induce IL6 release (Michaeloudes, Sukkar et al. 2011). Therefore TGFβ has the potential to either positively or negatively regulate IL6 protein synthesis as well as its signalling.

IL6 may also play a role in the regulation of TGFβ signalling. For example, IL6 positively regulates TGFβ induction of CTGF in studies of cardiac allograft rejection (Booth, Csencsits-Smith et al. 2010). In response to IL6 treatment in epithelial cells, TGFβ signalling was increased due to the reduced localisation of IL6 receptor with Calveolin-1 lipid rafts, this increased the level of TGFβ receptor localisation to early endosome antigen-1 (EEA-1) non lipid raft pathways, which meant an increase in trafficking of TGFβ receptors and increased TGFβ signalling (Zhang, Topley et al. 2005). However, IL6 was also shown to inhibit TGFβ stimulated apoptosis via PI3K/Akt and STAT3 in the liver (Chen, Chang et al. 1999). This indicates that there is a complex relationship between TGFβ and IL6; they are able to regulate each other's expression and signalling in both a positive and negative manner. Therefore there is a possibility that IL6 may be involved in the regulation of strain induced TGFβ signalling in some way. In addition, IL6 was increased and IL6R was decreased in both painful and ruptured tendon (Jelinsky, Rodeo et al. 2011; Legerlotz, Jones et al. 2012). This indicates that IL6 may also play a role in tendinopathy.

According to our TLDA analysis, IL6 mRNA showed a trend to increase after 2 hours in response to mechanical loading. Standard Taqman analysis showed IL6 was significantly regulated with strain compared to non-strained controls after 24 hours (data not shown). We have confirmed that at 24 hours IL6 stimulation is mediated via TGFβ signalling, as

TGFβRI inhibitor abrogated the strain induced increase in IL6 (data not shown). However this cannot be the case for the early stimulation because TGFβ activation does not occur until after 8 hours (see chapter 5), therefore these proteins may be involved in the activation of TGFβ. By using a functionally inhibiting antibody of IL6 as well as siRNA we aimed to characterise the role of IL6 in mechanotransduction.

In our TLDA experiments (see chapter 4) COX2 mRNA was increased in response to 2 hours mechanical loading, as this precedes TGFβ activation COX2 may be involved in the activation of TGFβ in response to mechanical loading. COX2 was also increased in both painful and ruptured tendon (Legerlotz, Jones et al. 2012), therefore COX2 may also play a role in tendinopathy. We chose to use an inhibitor known as Indomethacin, a non steroidal anti-inflammatory drug known to inhibit both COX1 and COX2, to determine whether COX2 was involved in mechanotransduction.

ADAM12 is reported to facilitate TGFβ signalling via the association of ADAM12 with TGFβRII, resulting in the accumulation of ADAM12/TGFβRII in the early endosome, the suppression of TGFβRII association with SMAD7 (inhibitory SMAD) and the initiation of SMAD signalling; which occurs in the absence of protease activity (Atfi, Dumont et al. 2007). ADAM12 is also able to bind to β1 and β3 integrins via a non-RGD and RGD-like motif respectively. ADAM12 ligation of integrin β3 in human breast carcinoma cells, can induce a RhoA signalling cascade followed by an increase in focal adhesion complexes (Kveiborg, Albrechtsen et al. 2008). As we have discussed in chapter 1, integrins have been reported as key sensors in the detection of mechanical load. The increase in ADAM12 in the response to mechanical load (see chapter 4) may induce the formation of focal adhesions and therefore increase the cells sensitivity to mechanical load or increase TGFβ signalling, which we have shown to be involved in mechanotransduction. Although as this evidence comes from experiments performed on human breast carcinoma and kidney cell lines, it may not be applicable to tenocytes. However there is a possibility that ADAM12 may play a role in the mechanotransduction pathway in tendon via integrin or TGFβ interaction. To determine whether ADAM12 is involved in TGFβ activation in response to mechanical loading we used siRNA to knockdown ADAM12 mRNA.

We have shown that TGFβ is activated in response to mechanical load. However the luciferase assay we use to determine TGFβ activation (See chapter 5) does not discriminate between the different isoforms of TGFβ. In order to determine which isoforms are

activated in response to mechanical strain we used siRNA to knockdown each isoform, by measuring the activation of TGFβ using the luciferase assay we could therefore determine the isoforms involved and by using qRT-PCR we can determine the differential effects of each TGFβ isoform upon strain induced gene expression.

SMAD4 is the co-SMAD involved in both TGFβ and BMP signalling (Shi and Massague 2003). We have shown that some genes such as MMP13 and ADAMTS5 are regulated with mechanical load but not via TGFβRI (see chapter 5). Therefore we chose to use siRNA to inhibit SMAD4 to determine whether SMAD4 signalling is involved in the regulation of these genes.

In summary, in this chapter we aimed to determine whether any of the above mechanisms are involved in either the activation of TGFβ or the regulation of metalloproteinase and matrix genes or both. Active and Total TGFβ were measured to confirm whether the inhibitors are abrogating the activation of TGFβ and a range of metalloproteinase and matrix genes were measured by qRT-PCR to confirm whether regulation occurs downstream of TGFβ activation.

7.2 METHODS

Inhibitor experiments: Tenocytes were seeded (1.5 x 10^6 cells/ml) in type I collagen (1mg/ml), plated into tissue train Flexcell™ plates and uniaxially strained at 5% at 1Hz for 48 hours with or without the following: Indomethacin, SB203580, Doramapimod, PD0325901, U-0126, Wortmannin and PI-103, GAP27, 18α-glycyrrhetinic acid, Thapsigargin, U73122, Nifedipine, Amiloride, Apyrase, recombinant IL6 (0.8ng/ml), IL6 antibody and LEAF™ purified rat IgG2 control antibody (for a summary of inhibitor functions and concentrations see table 7.1.) as appropriate (treatments were added just before the strain protocol was started). Medium was collected for analysis in the TGFβ Luciferase assay and gels were dissolved in Trizol for further RNA analysis. For full details of this protocol see methods section 2.2.

siRNA knockdown experiments: Tenocytes were incubated with transfection medium (TGFβ1, TGFβ2, TGFβ3, SMAD4, IL6 and ADAM12 siRNA plus Dharmafect 1 transfection reagent at 50nM and 0.2% respectively) for 48 hours. Cells were seeded (1.5 x 10^6 cells/ml) in type I collagen (1mg/ml), plated into tissue train Flexcell™ plates and uniaxially strained at 5% at 1Hz for 48 hours. Medium was collected for analysis in the TGFβ Luciferase assay and gels were dissolved in Trizol for further RNA analysis. For full details of this protocol see methods section 2.2.5.

RNA analysis: RNA was extracted using the tri-spin method essentially as described by (Ireland, Harrall et al. 2001). RNA was quantitated, reverse transcribed and analysed using qRT-PCR for a selection of metalloproteinase and matrix genes. Data was normalised to TOP1 and expressed as the $2^{\Delta Ct}$ or $2^{\Delta\Delta Ct}$. The Students T-test (two-way, paired) was used to assess statistical significance. In some experiments we were unable to perform statistical analysis on experiments where $n<3$. For full details of this protocol see methods section 2.3.

TGFβ Luciferase assay: The TGFβ luciferase assay was conducted essentially as described by Jonk (Jonk, Itoh et al. 1998). In brief, TGFβ was measured using a cell based system to measure SMAD activity, a key step in canonical TGFβ signalling. Medium from 48 hour strained gels was transferred onto SW1353 cells transfected with a SMAD specific construct linked to a luciferase reporter. Total TGFβ was measured by heat activating the TGFβ in the medium before incubation with the SW1353 cells. Luciferin was added to

samples and fluorescence measured on the illuminometer. For details of this protocol see methods section 2.4.1.

Inhibitor	Concentration	Function
Indomethacin	25µM	Inhibitor of COX1 and COX2 (Wang, Jia et al., 2003)
SB203580	5µM	Inhibitor of p38 MAPK (Bain, Plater etal., 2007)
Doramapimod	0.1µM	Inhibitor of p38 MAPK (Bain, Plater etal., 2007)
PD0325901	0.1µM	Inhibitor of MKK1 (Bain, Plater etal., 2007)
U-0126	20µM	Inhibitor of MKK1 (Bain, Plater etal., 2007)
Wortmannin	0.1µM	Inhibitor of PI3K (Bain, Plater etal., 2007)
PI-103	0.5µM	Inhibitor of PI3K (Bain, Plater etal., 2007)
GAP27	50µM	Specific inhibitor of Cx43 Gap junctions (Wright, Van Steensel et al., 2009)
18α-glycyrrhetinic acid	100µM	Broad spectrum Gap junction inhibitor (Kamijo, Haraguchi et al., 2006)
Thapsigargin	10µM	Non-competitive inhibitor of calcium ATPase (Law, Pachter et al., 1990)
U73122	10µM	Inhibitor of phospholipase C (Suh, Kim et al., 2000)
Nifedipine	10µM	An L-type Calcium channel blocker (Hattori, Matsunaga et al., 2006)
Amiloride	10µM	A T-Type calcium channel blocker (Rusch, Kros et al., 1994)
Apyrase	10U/ml	Contains ATPase and ADPase which catalyse the degradation of ATP and ATP respectively (Cao, young et al., 2007)
IL6 antibody	0.4µg/ml	Functionally inhibiting antibody against IL6 (Antibody requirement calculated according to level of IL6 measured in the media)
LEAF™ purified rat IgG2 control antibody	0.4µg/ml	Control antibody for functional studies

Table 7.1 Summary of Inhibitors and their functions. References listed in the right hand column are those used to determine concentration for in the current study (Law, Pachter et al. 1990; Rusch, Kros et al. 1994; Suh, Kim et al. 2000; Wang, Jia et al. 2003; Hattori, Matsunaga et al. 2006; Kamijo, Haraguchi et al. 2006; Bain, Plater et al. 2007; Cao, Young et al. 2007; Wright, van Steensel et al. 2009).

7.3. RESULTS

7.3.1. Inhibition of Calcium signalling

Active TGFβ was increased and total TGFβ remained unchanged in response to mechanical loading as we have reported in previous experiments (Figure 7.1 A). α18-glycerritinic acid induced a trend to decrease in the level of active TGFβ in strained and non-strained cultures, with no effect upon total TGFβ. Whereas gap27 has no effect upon active TGFβ in terms of either active or total TGFβ. Thapsigargin induced a trend to decrease in the level of active TGFβ with mechanical loading but also decreased total levels of TGFβ in both strained and non-strained cultures. U73122, Nifedipine and Amiloride did not have substantial effects upon the levels of active or total TGFβ released in response to mechanical loading.

ADAM12 mRNA is increased with mechanical loading as in previous experiments (Figure 7.1 B). Thapsigargin induced a trend to decrease in the levels of ADAM12 in both strained and unstrained cultures. U73122 and α18-glycerritinic acid induced a trend to increase in ADAM12 expression in non-strained cultures and decrease in strained cultures. Gap27, nifedipine and amiloride did not have an effect upon the regulation of ADAM12 with mechanical load.

In this experiment Elastin was increased (~1.5 fold) with mechanical load, substantially less than reported previously (Figure 7.1 C). U73122 and Thapsigargin reduced the overall level of Elastin expression with no effect upon the strain response (fold change with strain). α18-glycerrhitinic acid induced a trend to decrease in the strain induced regulation of Elastin. Gap27 and nifedipine had little effect upon the strain regulation of elastin, however amiloride increased the strain induced response by decreasing Elastin expression in non-strained cultures only.

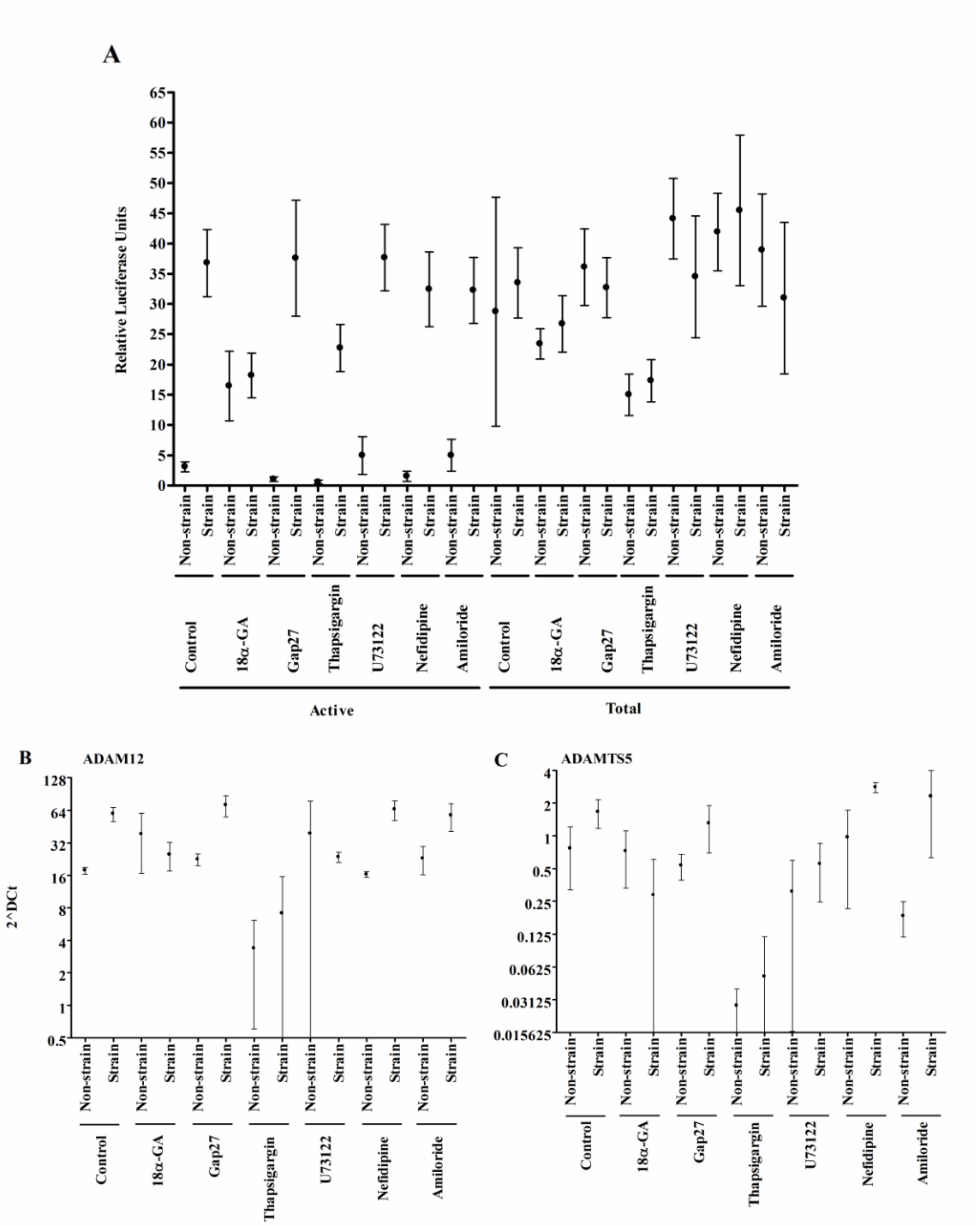

Figure 7.1. Inhibition of Calcium signalling in mechanically strained tenocytes. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). GAP27 (50µM), 18α-glycyrrhetinic acid (100µM), Thapsigargin (10µM), U73122 (10µM), Nifedipine (10µM), Amiloride (10µM) were added to cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml before they were cyclically strained at 5% at 1Hz for 48 hours. Data was normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=2, n=1 is shown and is representative of both sets of data, ± SD). (B and C) Quantitative Real Time PCR analysis of ADAM12 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain (1Hz) after 48 hours. Data normalised to TOP1 and expressed as the $2^{\Delta Ct}$ (n=2, n=1 is shown and is representative of both sets of data, ± SD).

7.3.2. ATP/ADP degradation

Apyrase decreases the level of active TGFβ increased with mechanical load, however the total level of TGFβ was also decreased in both strained and non-strained samples (Figure 7.2 A). In order to test whether Apyrase effects the measurement of TGFβ using the luciferase assay SW1353 cells were incubated with 1ng/ml TGFβ and Apyrase (Figure 7.2 B). The results showed that Apyrase inhibited the detection of TGFβ in the medium. We also measured a range of metalloproteinases and cytokines using qRT-PCR including TGFβ1, TGFβ2, TGFβ3, IL1, IL6, MMP1, ADAM12 and Elastin (Figure 7.2 C). Overall, Apyrase affected basal levels of gene expression but had no clear affects upon strain regulation of the genes tested

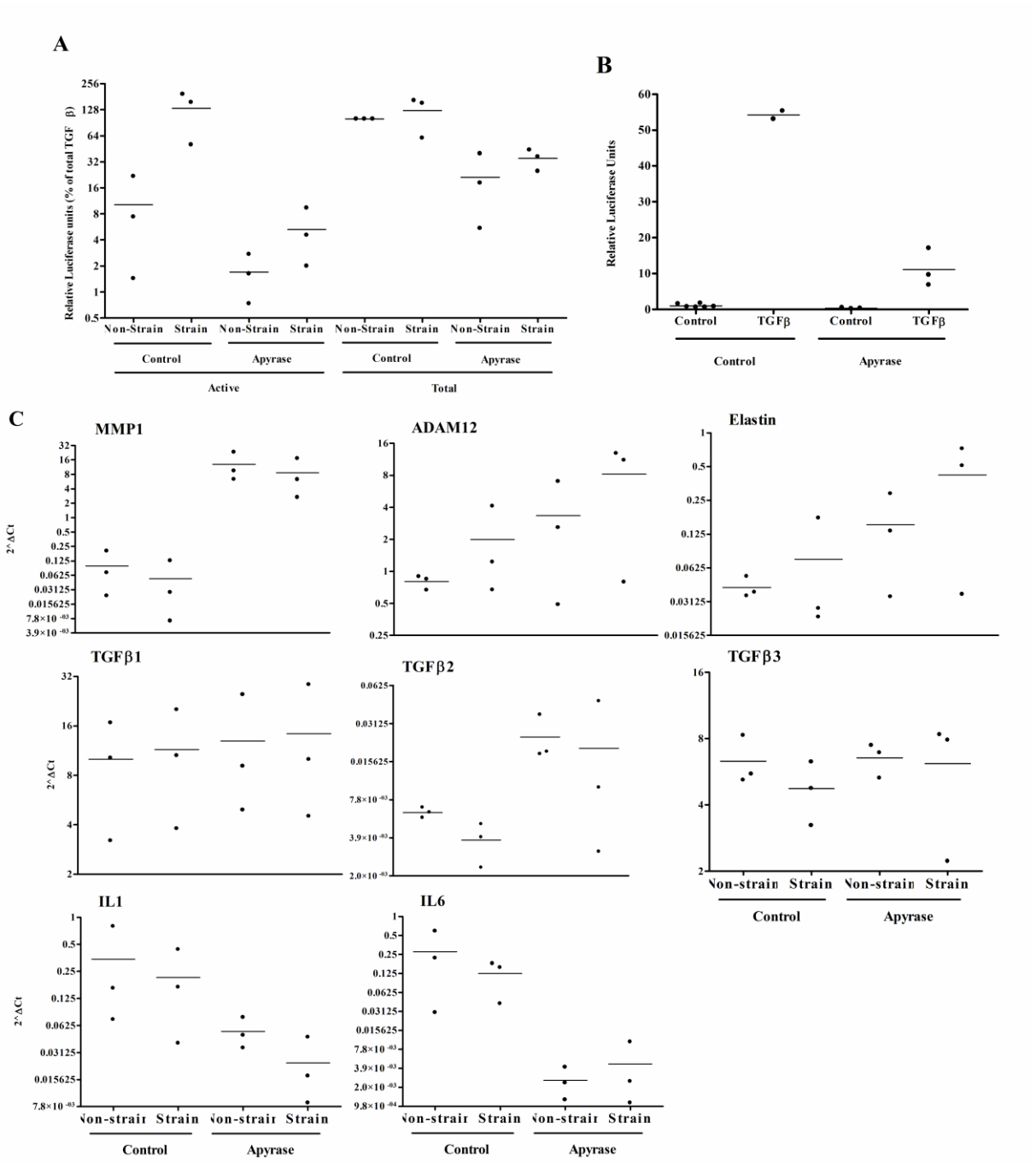

Figure 7.2. Apyrase regulates basal levels of cytokines, protease and matrix genes but has no clear effect upon their strain regulation. (A,B) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Apyrase was added before the straining protocol (10U/ml). Data is normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=3). (B) TGFβ ±Apyrase were added to the luciferase assay to test whether Apyrase effects the detection of TGFβ. (C) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the $2^{\Delta Ct}$ (n=3).

7.3.3. Inhibition of Kinases

The responses of two cell isolates are shown in figure 7.3 A and B, active TGFβ was increased with strain as we have previously reported. SB203580, Doramapimod, PD0325901, Wortmannin and PI-103 had little effect upon the strain induced up-regulation of active TGFβ in either cell isolate. U0126 induced a trend to decrease in the level of active TGFβ measured in strained cultures. However there was a high standard deviation in these experiments and high variability between cell isolates.

MMP1 mRNA was decreased with mechanical loading in control cultures (Figure 7.3 C). The p38 MAPK inhibitors SB203580 and Doramapimod did not modify MMP1 regulation with strain. MKK1 pathway inhibitors were inconsistent in their response. PI3K inhibitors PI-103 increased MMP1 mRNA in both strained and non-strained conditions (p=0.03), however despite the addition of PI-103 there was still a decrease in the level of MMP1 in strained compared to non-strained cultures.

MMP13 and ADAMTS5 were decreased and increased respectively in response to strain as in previous experiments (Figure 7.3 D and E). However the addition of MAPK inhibitors did not significantly regulate strain induced MMP13 or ADAMTS5 expression.

Elastin was increased with mechanical strain as we have shown previously. SB203580, Doramapimod, Wortmannin and PD0325901 had little effect upon the strain regulation of Elastin (Figure 7.3 F). U0126 increased the level of Elastin in non-strained cultures compared to untreated controls but had no effect upon strained samples (p=0.04) and PI-103 decreased the level of Elastin in strained cultures compared to untreated controls (p=0.005). However the strain response (as a fold change) was not substantially affected with the addition of U0126 or PI-103.

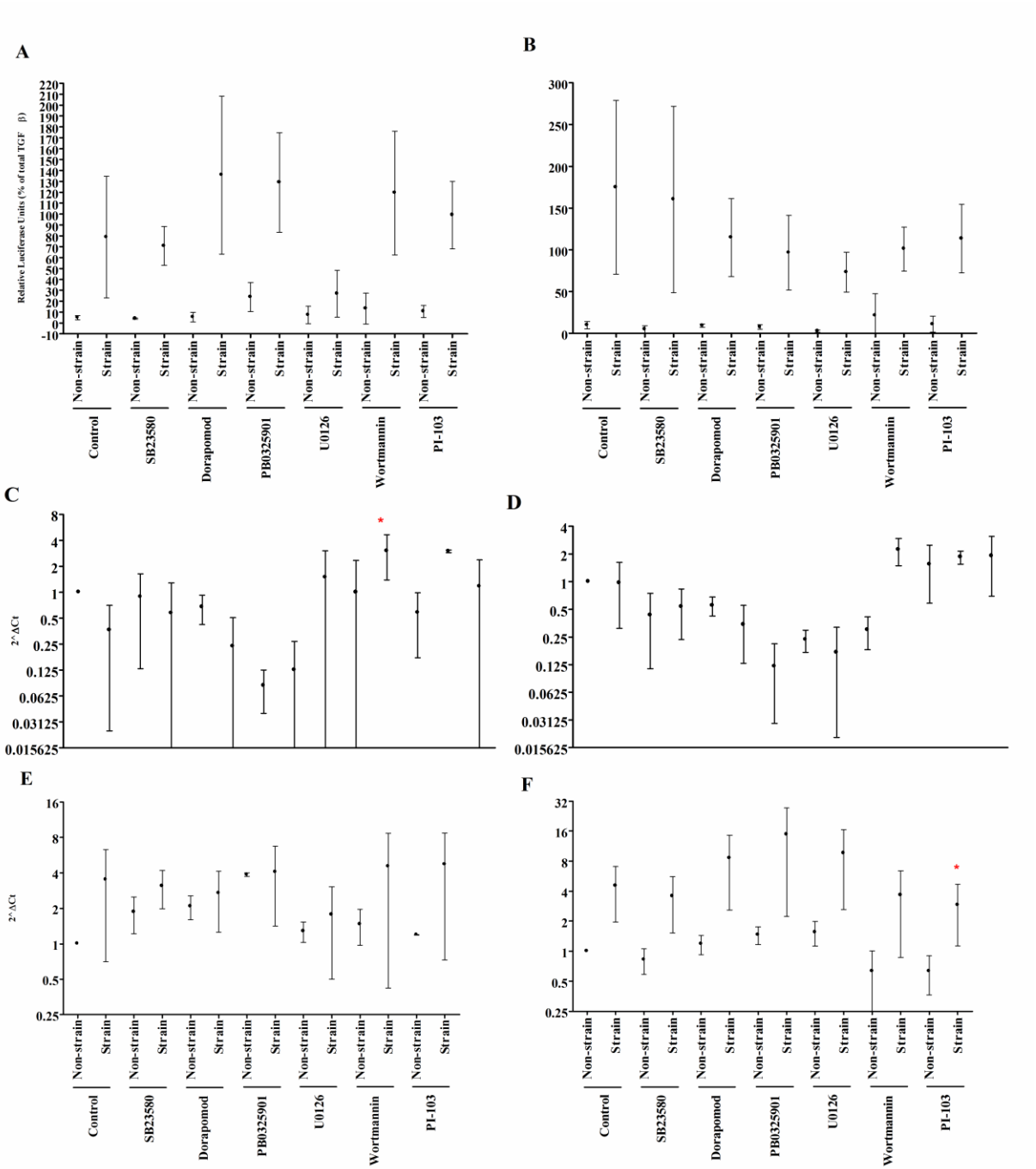

Figure 7.3. Inhibition of kinases in mechanically strained tenocytes. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). SB203580 (5µM), Doramapimod (0.1µM), PD0325901 (0.1µM), U-0126 (20µM), Wortmannin (0.1µM) and PI-103 (0.5µM) were added to cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml before they were cyclically strained at 5% at 1Hz for 48 hours. Data was normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=2, data of n=1 is shown and is representative of both repeats, ± SD). (B-E) Quantitative Real Time PCR analysis of MMP1 (B), MMP13 (C), ADAMTS5 (D) and Elastin (E) in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data normalised to TOP1 and expressed as the $2^{\Delta Ct}$ ± SD. Students T-test (two way, paired) was used to test statistical significance. Significance is denoted by * (compared to paired [either non-strain or strain] control) (n=3).

7.3.4. COX inhibition

Active TGFβ was increased with 48 hours of strain as we have reported previously (Figure 7.4 A). Indomethacin had no effect upon the strain regulation of TGFβ, with no change in active or total TGFβ in strained or non-strained cultures. MMP1 and MMP13 were decreased with mechanical strain and COL1A1, ADAMTS5 and Elastin were increased in response to loading, however these levels were not as marked as in previous experiments. The addition of Indomethacin had no effect on the expression of these genes in response to strain (Figure 7.4 B).

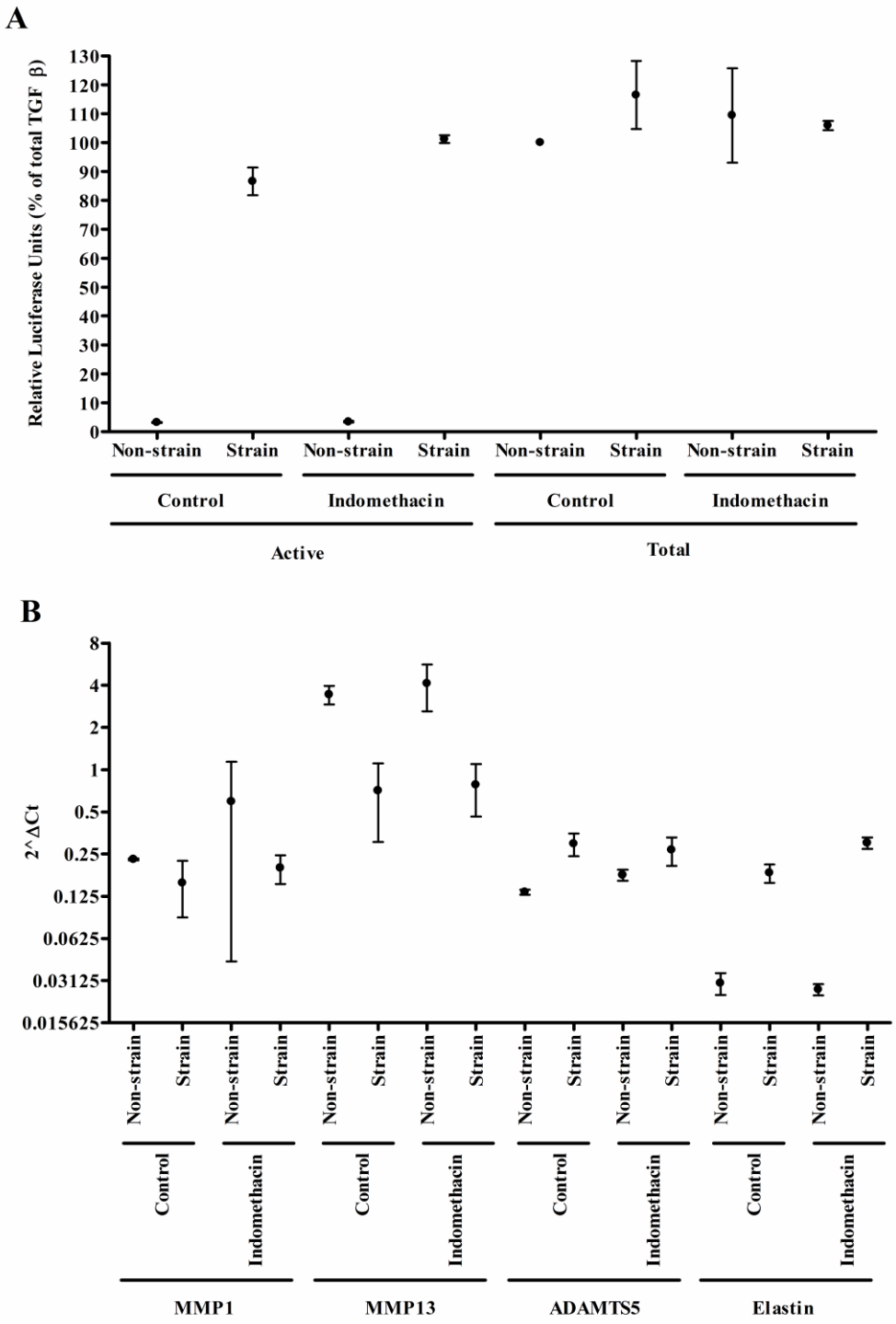

Figure 7.4. Cyclooxygenase inhibition has no effect upon the level of TGFβ activation stimulated by strain. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Indomethacin (25μM) was added to cultures before the straining protocol. Data was normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=2, n=1 is shown and is representative of the two repeats, ± SD). (B) Quantitative Real Time PCR analysis of MMP1, MMP13, ADAMTS5, COL1A1 and Elastin in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data was normalised to TOP1 and expressed as the $2^{\Delta Ct}$ ± SD (n=1 is shown and is representative of the two repeats).

7.3.5. IL6 functionally inhibiting antibody inhibits TGFβ activation

Active TGFβ was increased in response to mechanical loading as previously reported. IL6 alone had no effect upon activity of TGFβ; however, the IL6 antibody decreased the level of active TGFβ in strained cultures by approximately ~50% (Figure 7.5 A). The addition of recombinant IL6 in combination with IL6 neutralising antibody did not affect the level of active TGFβ compared to non-treated controls. Detection of total TGFβ was higher in controls than in cultures treated with recombinant IL6 or IL6 neutralising antibody.

MMP1 mRNA was decreased with mechanical loading as previously reported (Figure 7.5 B). Recombinant IL6 had little effect upon MMP1 expression; however IL6 neutralising antibody inhibited the decrease in MMP1 in response to strain. MMP13 mRNA was decreased with strain (Figure 7.5 D); recombinant IL6 had little effect upon strain regulation of MMP13 mRNA; however a combination of recombinant IL6 and IL6 antibody increased the level of MMP13 in strained cultures. IL6 antibody alone had little effect upon strain regulation of MMP13. ADAMTS5, Elastin and COL1A1 were increased with mechanical loading as in previous experiments (Figure 7.5 B, C and E). Addition of recombinant IL6 and IL6 neutralising antibody had no effect upon the expression of ADAMTS5, Elastin or COL1A1.

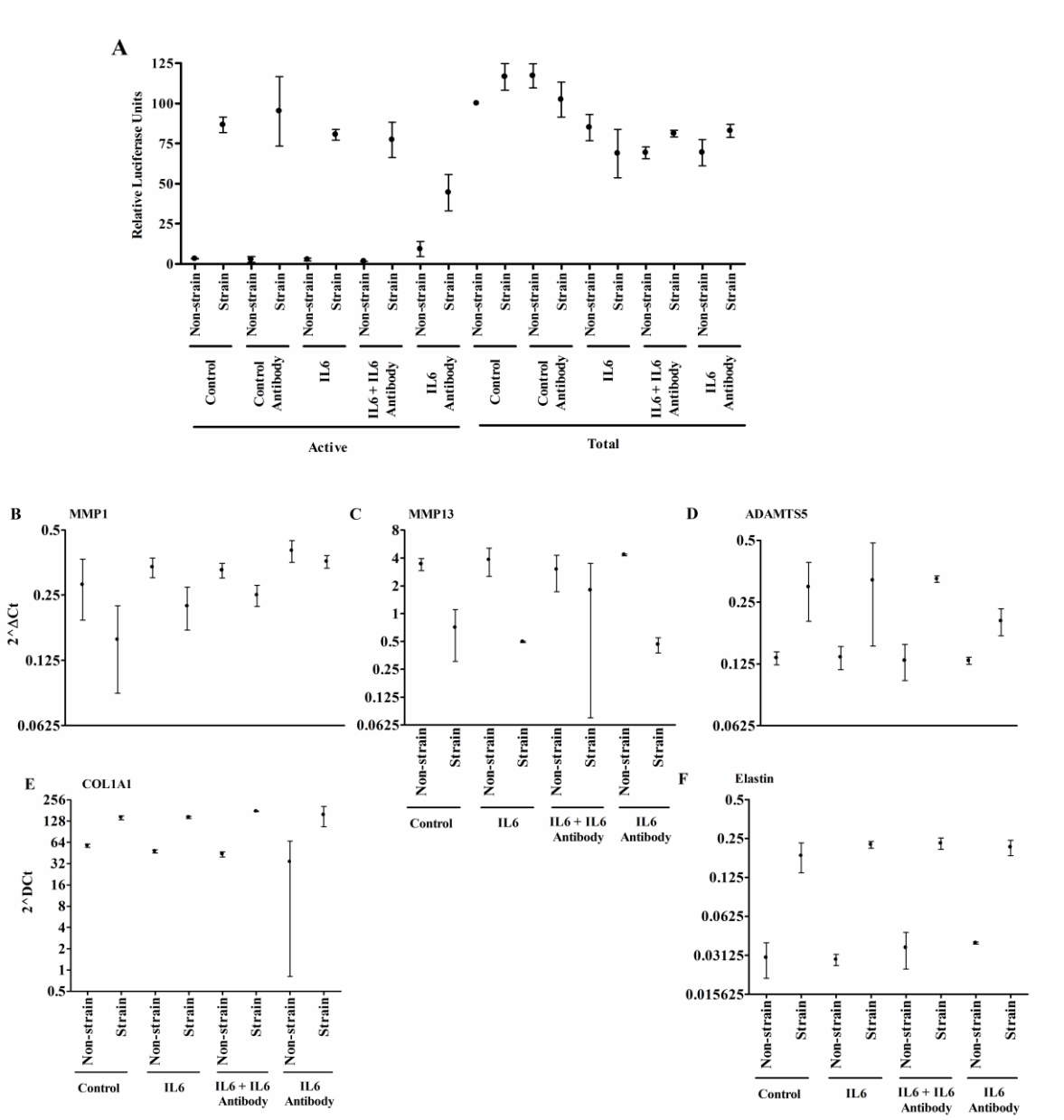

Figure 7.5. IL6 inhibitory antibody reduces the level of TGFβ activation stimulated by strain. (A) A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). 0.8ng/ml recombinant IL6 and 0.4µg/ml IL6 antibody were added to cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml before they were cyclically strained at 5% at 1Hz for 48 hours. Data was normalised to transfection controls and negative controls before expression as a percentage of Total TGFβ (non-strain) (n=2, n=1 is shown and is representative of the two repeats, ± SD). (B-F) Quantitative Real Time PCR analysis of MMP1 (B), MMP13 (D), ADAMTS5 (C), COL1A1 (E) and Elastin (F) in cells seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml after 5% cyclic strain after 48 hours. Data was normalised to TOP1 and expressed as the $2^{\Delta Ct}$ ± SD (n=2, n=1 is shown and is representative of the two repeats).

7.3.6. siRNA Knockdown

TGFβ isoforms

All TGFβ siRNA produced 99% knockdown of their respective isoforms (Figure 7.6 A). TGFβ1 and TGFβ3 siRNA reduced the level of TGFβ activation by 71 and 39% respectively (Figure 7.6 B). TGFβ2 siRNA had little effect upon the level of TGFβ activation in response to mechanical strain. TGFβ1 siRNA induced a trend to increase, decrease and decrease in MMP1, ADAMTS5 and TGFβ2 expression (respectively) in strained cultures compared to the scrambled control (Figure 7.7). TGFβ2 siRNA induced a trend to decrease in MMP13 and ADAMTS5 expression in strained cultures compared to scrambled controls. TGFβ3 siRNA induced a trend to decrease in MMP1 and MMP13 expression in strained cultures compared to scrambled controls. TGFβ siRNA had no clear effects upon Elastin regulation (Data not shown).

ADAM12

ADAM12 siRNA successfully knocked down the expression of ADAM12 by 99% (Figure 7.6 A). However ADAM12 siRNA had no effect upon TGFβ activation or the regulation of MMP1, MMP13, Elastin, ADAMTS5, SMAD4, TGFβ1, TGFβ2 or TGFβ3 mRNA expression (Figure 7.7) (Elastin, TGFβ1, TGFβ2, TGFβ3 and SMAD mRNA data not shown).

IL6

We were unable to determine whether IL6 was knocked down was successful, as IL6 was undetectable in all samples. However, previous knockdown was more than 99% successful. IL6 knockdown induced a trend to increase in MMP1 and MMP13 expression in strained samples compared to scrambled controls (~2fold) (figure 7.7 A and B). IL6 knockdown had no effect upon ADAM12, ADAMTS5, Elastin, SMAD4, TGFβ1, TGFβ2 or TGFβ3 mRNA expression. (Elastin, TGFβ1, TGFβ2, TGFβ3 and SMAD mRNA: data not shown).

SMAD4

SMAD4 mRNA was successfully knocked down with siRNA (99%) (Figure 7.6 A). SMAD4 knockdown induced a trend to increase in MMP13 expression (figure 7.7 B) and a trend to decrease in ADAM12 expression (data not shown) in response to strain compared to the scrambled siRNA control. SMAD4 siRNA had little effect upon MMP1, Elastin (data not shown) and ADAMTS5 regulation with mechanical strain (Figure 7.6 A and C).

A

	Percentage Knock Down
TGFβ1 siRNA	99.99195835
TGFβ2 siRNA	99.98887666
TGFβ3 siRNA	99.85329901
SMAD4 siRNA	99.88446913
ADAM12 siRNA	99.88580501

B

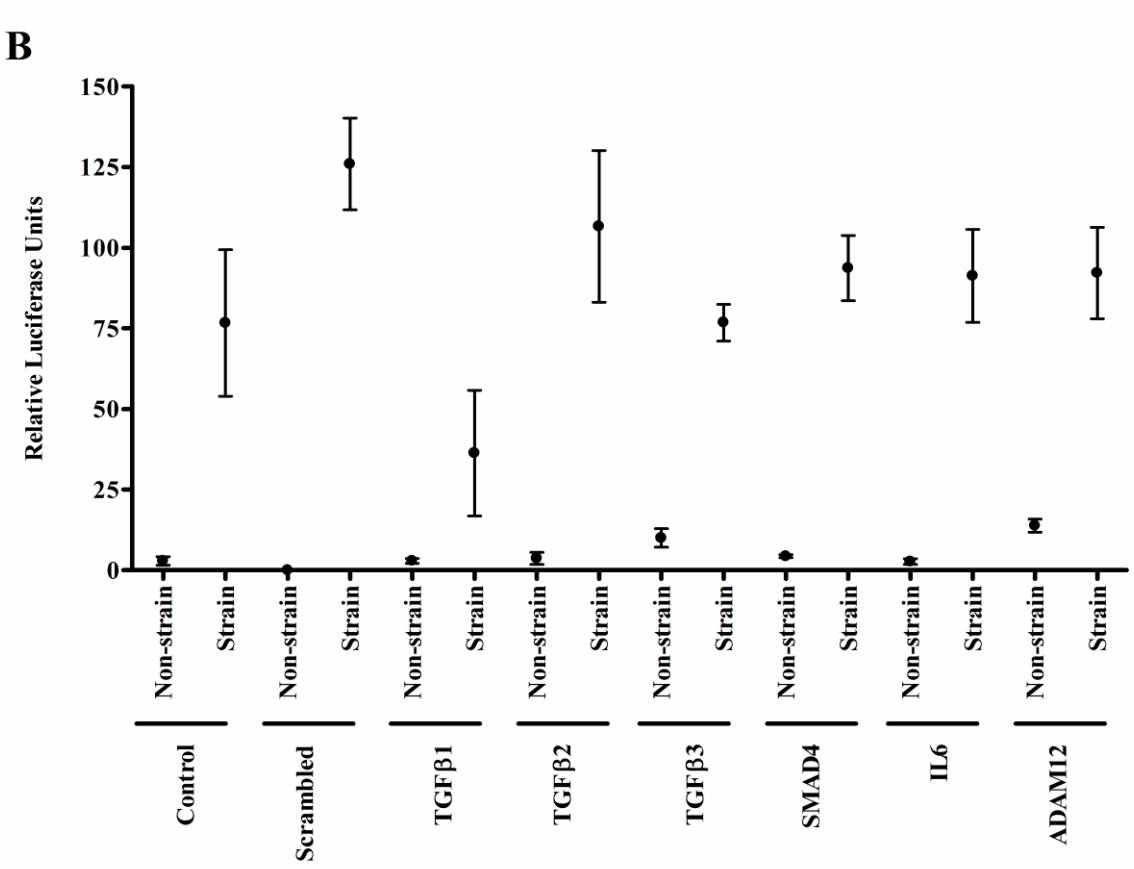

Figure 7.6. Knockdown of TGFβ1 and TGFβ3 RNA reduces the activation of TGFβ with strain. Percentage knockdown of TGFβ isoforms 1, 2 and 3, SMAD4 and ADAM12 are shown (A). A cell based luciferase assay measuring levels of SMAD activatory soluble factor (see methods for details). Cells were treated with transfection media for 48 hours before they were seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml. Tenocyte seeded gels were cyclically strained at 5% at 1Hz for 48 hours. Data was normalised to transfection controls and negative controls before expression as a percentage of total TGFβ (non-strain) (n=1, ± SD) (B).

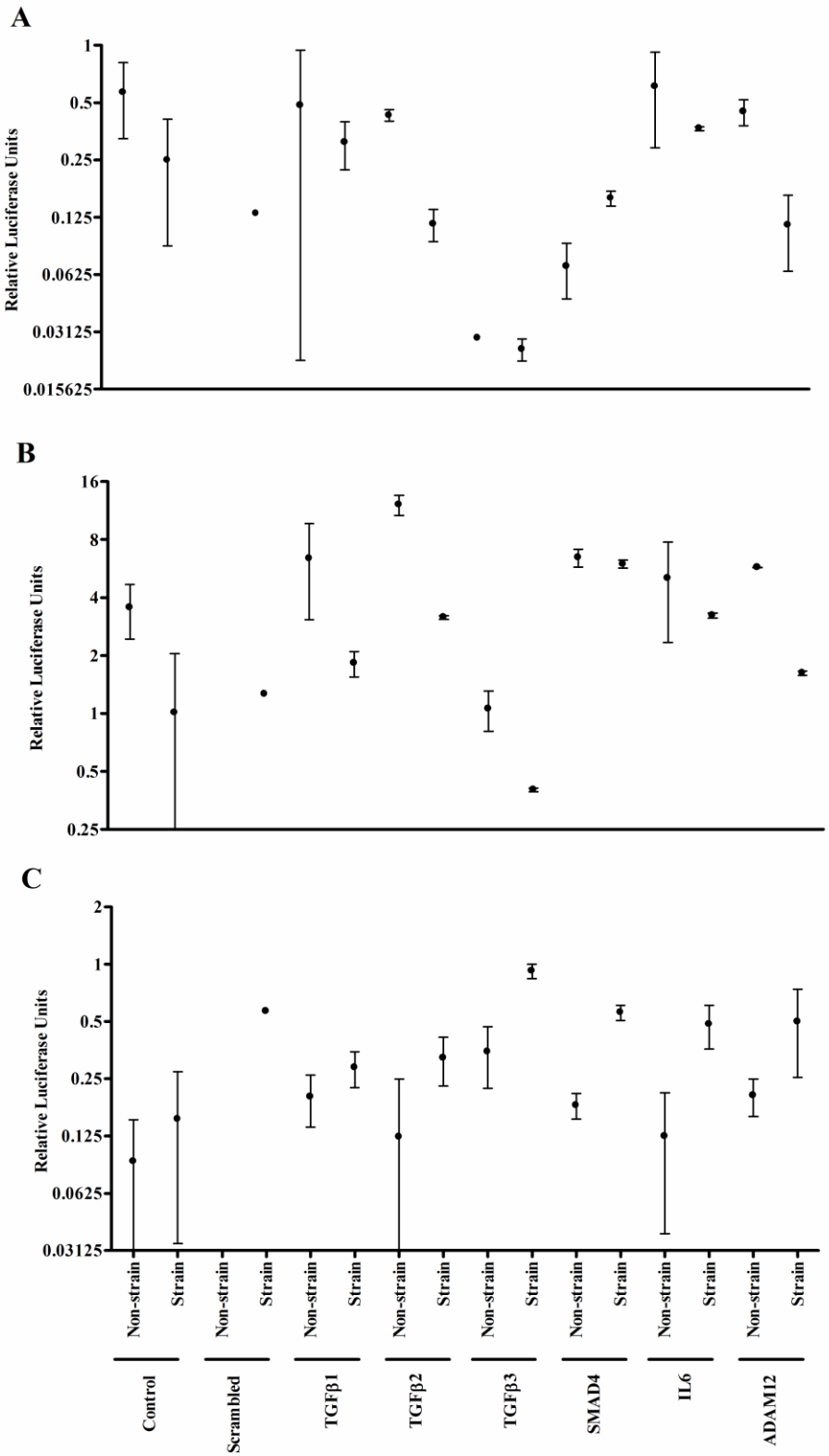

Figure 7.7. siRNA knockdown and regulation of gene expression in response to strain. Cells were treated with transfection media for 48 hours before they were seeded at $1.5x10^6$ cells/ml in type I rat tail collagen at 1mg/ml. Tenocyte seeded gels were cyclically strained at 5% at 1Hz for 48 hours. Quantitative RT-PCR was used to measure MMP1 (A), MMP13 (B) and ADAMTS5 (C) RNA. Data is normalised to TOP1 and expressed as $2^{\Delta Ct} \pm SD$ (n=1).

7.4. DISCUSSION

In this chapter I present preliminary evidence to indicate that TGFβ1 and TGFβ3 are the major isoforms of TGFβ activated with mechanical strain (approximately 70% and 30% respectively). This correlates well with the mRNA data, which showed high levels of expression of TGFβ1 and TGFβ3 compared to TGFβ2 (TGFβ1, TGFβ2 and TGFβ3 had an average Ct of 22.5, 33.3 and 25.6 respectively). We have evidence to show that MMP1, MMP13 and ADAMTS5 are regulated via separate pathways, and that each involves TGFβ signalling in some way (discussed further below). In an effort to further characterise preceding events in the activation of TGFβ, we have shown that calcium signalling via GAP junctions, phospholipase conversion of PIP_2 to PI_3 kinase, as well as the transport of calcium via L and T type calcium channels are probably not involved in the activation of TGFβ with strain or proceeding gene regulation. MAP kinase signalling pathways previously thought to be involved in TGFβ signalling crosstalk (P38 MAPK, MKK1 and PI3K kinase signalling pathways) are also not involved in the strain induced activation of TGFβ or consequent gene regulation. And despite the increase in COX2 mRNA expression preceding TGFβ activation, prostaglandin is not involved in the activation of TGFβ or gene regulation in response to mechanical load. An IL6 functionally inhibiting antibody did reduce the activation of TGFβ by ~50%, and although the activation was not completely abrogated, this indicates that IL6 may play a role in the activation of TGFβ in response to mechanical load. In apparent contradiction, IL6 knockdown showed no effect on activation of TGFβ, but did show an effect on MMP1. This was similar to the effect seen in MMP1 with the IL6 inhibitory antibody. This suggests that IL6 does have some effect upon the regulation of MMP1 in response to mechanical load. However to confirm this more repeats and further pathway analysis is required.

We were unable to determine the direct effect of Thapsigargin, 18α-glycyrrhetinic acid or Apyrase upon TGFβ activity, due to their inhibitory effects upon this assay. Apyrase addition to the cell based luciferase assay confirmed this, however due to time constraints we were unable to repeat this for all inhibitors. It has previously been reported that Thapsigargin interferes with the TGFβ luciferase assay and therefore yields an inaccurate measure of TGFβ (Wicks, Lui et al. 2000). It is interesting that 18α-glycyrrhetinic acid, Thapsigargin and Apyrase, which are all inhibitors of calcium signalling, affected the

efficiency of the TGFβ luciferase assay to detect TGFβ. This indicates that at least in SW1353 chondrosarcoma cell line calcium signalling may be involved in the regulation of the TGFβ signalling pathway. Wicks et al, have reported that increased cytoplasmic calcium can regulate SMAD function through the activity of Cam Kinases (Wicks, Lui et al. 2000). This shows that calcium signalling can regulate TGFβ signalling. However in the current tendon study this is less clear.

In terms of mRNA regulation in response to the addition of the range of calcium inhibitors we see little change in the strain response of ADAM12 and Elastin expression in response to Gap27, amiloride or nifedipine. The addition of Thapsigargin decreased ADAM12 and Elastin in both strained and non-strained cultures. This indicates that increased cytosolic calcium has a dampening effect upon Elastin and ADAM12 gene expression which may be a reflection of cell viability. Therefore cell viability testing in the presence of thapsigargin must be completed in order to validate these experiments. Even though α18-glycerrhitinic acid decreased strain induced response in TGFβ, Elastin and ADAM12, the effect may be non-specific to connexins as it is not seen with Gap27. Although it is possible that another connexin is involved. U73122 increased ADAM12 expression in non-strained cultures, decreased ADAM12 expression in strained cultures and decreased overall expression of Elastin, in the absence of any differential regulation of TGFβ. This may be due to non-specific effects of U73122, rather than its primary function of inhibiting Phospholipase C. Other functions of U73122 include activation of ion channels, release of calcium from intracellular stores and induction of IP_3 mediated calcium release (Mogami, Lloyd Mills et al. 1997). The lack of a clear abrogation of strain induced effects indicates that another pathway may be affecting ADAM12 and Elastin expression independent of TGFβ signalling or the strain response.

Overall this range of calcium inhibitors does not show any involvement of calcium signalling in the regulation of either TGFβ or gene regulation of ADAM12 or Elastin in response to mechanical strain. The literature reports varied strain responses in different species in terms of calcium transients. For example studies on cells from human flexor digitorum profundus tendon have shown that both fluid flow (Elfervig, Francke et al. 2000; Franke, Banes et al. 2000) and static elongation (4-6%) (Elfervig 2001; Wall and Banes 2005) induce an increase in calcium. A canine ligament study showed fluid flow (25dynes/cm^2) also induced significant calcium transients (Hung, Allen et al. 1997). However in avian and rodent tendon cell studies there was no effect of 5% cyclic loading

(Wall and Banes 2004) or fluid flow (Elfervig, Lotano et al. 2001; Archambault, Elfervig-Wall et al. 2002). Therefore, the effects appear to be variable, as studies of human and canine but not avian and rodent species produced calcium transients in response to mechanical load. Although we have not confirmed whether calcium signalling is stimulated in response to mechanical strain in the current study, it does not seem to have an effect upon TGFβ activation and gene regulation in response to strain. Further study may involve the measurement of calcium transients in response to mechanical load in our system. Additional study in this area could help us to understand what stimuli is required to induce calcium signalling in response to mechanical strain and which types of cells are able to respond to mechanical strain in this way. In addition if our cells are capable of producing a calcium signal in response to mechanical strain, what changes occur as a result of this? The literature shows that collagen synthesis as well as DNA synthesis is stimulated in response to mechanical loading through calcium signalling via GAP junctions (Banes, Weinhold et al. 1999; Sood, Bynum et al. 1999). However, no direct evidence implicates calcium signalling in the regulation of metalloproteinases with strain. Therefore to confirm that collagen is regulated via calcium signalling, measurement of collagen mRNA in response to strain must be completed. It may be that calcium signalling specifically regulates collagen, is not involved in MMP regulation and that other genes are regulated via a different pathway.

We were unable to measure TGFβ using the luciferase assay in cultures where Apyrase was added to the culture medium. However, we could use another means of TGFβ measurement (i.e. ELISA) to confirm whether Apyrase regulates TGFβ activity or protein levels in future experiments. Apyrase caused differential expression of key metalloproteinase and matrix genes as well as cytokines associated with tendon disease (Figure 7.2); however these changes did not result in the abrogation of strain induced regulation of these genes. MMP1, ADAM12, Elastin, IL1 and IL6 showed a trend to increase, TGFβ1 and TGFβ3 were not substantially regulated and TGFβ2 showed a trend to decrease in response to Apyrase. All of these genes showed a similar pattern of regulation in response to strain with or without Apyrase (i.e. the fold change with strain remained the same), except for IL6 which showed a trend to decrease in response to strain. However we cannot say that Apyrase abrogated the strain response due to the large stimulation of IL6 with Apyrase, in addition the changes induced by apyrase were not statistically significant. IL1 is a known stimulator of IL6 expression (Sironi, Breviario et

al. 1989), therefore the increase in IL1 in response to Apyrase treatment may be responsible for the induction of the IL6 response. This could be tested by the use of an IL1 receptor antagonist. In rodent Achilles tendon studies P2Y1 and P2Y2 purinoceptors were shown to be responsible for downstream ATP signalling events (increase in intracellular calcium) (Fox, Jones et al. 2005; Jones, Yang et al. 2005). Therefore the use of more specific inhibitors of purinoreceptor signalling may be used to confirm these results. For example; Suramin, a P2 purinoreceptor inhibitor and CGS-21680, a P1 and adenosine purinoreceptor agonist could be used to isolate specific purinoreceptors.

Inhibition of p38 MAPK signalling was achieved with the addition of SB203580 and Doramapimod. There was no substantial decrease in the level of TGFβ activated in response to mechanical loading. In addition there was no clear difference in the strain induced regulation of MMP1, MMP13, ADAMTS5 or Elastin. ADAMTS5 was increased in response to both of these inhibitors, although this did not affect the level of expression with strain. This suggests that p38 MAPK signalling does not play a role in the activation of TGFβ or regulation of gene expression in response to mechanical load. However, since activity of the kinase inhibitors was not confirmed, we cannot rule out some role for these pathways. To confirm this we could use western blotting of the phosphorylated and non-phosphorylated forms of p38 from protein extracts of cells.

Inhibition of MMK1 pathway signalling was achieved using the PD0325901 and U0126 inhibitors. There was no substantial decrease in the level of TGFβ activated in response to mechanical load in response to PD0325901; however there was a decrease in active TGFβ when U0126 was added. Differential effects of these two inhibitors suggest that the U0126 inhibitor is showing non-specific affects. U0126 increased the expression of MMP1 and decreased the expression of MMP13 in both strained and non-strained cultures. In addition, the strain induced increase in ADAMTS5 was abrogated in response to U0126. As this effect was only observed in response to U0126 and not PD0325901, this must be an unspecific effect unrelated to MKK1. This suggests that MKK1 is not involved in the mechanical activation of TGFβ or the regulation of MMP1, MMP13, ADAMTS5 or Elastin. As these inhibitors did produce changes in gene regulation, it appears that they do have some effect, although confirmation that the MKK1 pathway is specifically abrogated would further substantiate this negative data.

In order to inhibit PI3K signalling Wortmannin and PI-103 inhibitors were chosen. There was little affect upon the strain mediated TGFβ activation, in addition the response of MMP1, MMP13, ADAMTS5 and Elastin in response to mechanical loading remained unchanged. This suggested that PI3K is not involved in the activation of TGFβ in response to mechanical loading. In order to confirm these negative results, the efficacy of these inhibitors must be substantiated. This could be achieved by western blotting of protein extracts for PI3K.

The inconsistency between pairs of inhibitors chosen for the inhibition of p38, MKK1 and PI3K, as well as the lack of abrogation of strain induced changes indicates a high probability that these pathways are not involved in the strain response. Published data indicates that p38 is increased in response stress deprivation in rabbit tendon (Lambert, Colige et al. 2001; Kawabata, Katsura et al. 2009). As we have shown that p38 may not be involved in the strain regulation of selected genes, this indicates that different pathways are activated in response to mechanical load and stress deprivation.

Indomethacin is an NSAID that inhibits both COX1 and COX2. Previously we showed that COX2 was increased in trend after 2 hours in the presence of mechanical loading. Therefore we wanted to test whether the inhibition of COX2 would decrease the level of TGFβ activation in response to mechanical loading, we could then confirm whether COX2 preceded and regulates TGFβ activation in response to mechanical load. We were also interested in COX2 as it is increased in tendinopathy (Legerlotz, Jones et al. 2012). However we have shown that neither the activation of TGFβ in response to mechanical load nor the regulation of metalloproteinase and matrix genes in response to load is abrogated by the inhibition of COX. Therefore COX2 is not involved in the regulation of TGFβ and matrix genes in response to mechanical load. However, in order to confirm that COX2 activity is abrogated by indomethacin in these experiments we could measure PGE_2 by western blotting or ELISA; As COX2 regulates the production of prostaglandins.

We have shown that IL6 functionally inhibiting antibody reduces the activation of TGFβ by ~50%. We have also shown that the decrease in MMP1 seen in response to mechanical loading is abrogated with the addition of this same antibody. However none of the other genes that were analysed show the same response. As MMP13 and ADAMTS5 are not regulated via canonical TGFβ signalling we would not expect these genes to be regulated by IL6 siRNA. Elastin and COL1A1 are regulated via TGFβR1 (see chapter 4), but the

strain induced regulation of these genes was not inhibited by IL6 siRNA. This may be due to the sensitivity of these genes to TGFβ stimulation, i.e. the threshold of TGFβ signalling required to stimulate changes in gene regulation may be lower. Despite the effect of the IL6 antibody in reducing the level of active TGFβ this does not account for all TGFβ activation stimulated in response to mechanical loading. Therefore another mechanism may be involved. However IL6 inhibitory antibody experiments were only repeated on two cell isolates, therefore more repeats are required to confirm this. In addition siRNA knockdown of IL6 did not show the same decrease in TGFβ activation seen with the functionally inhibiting antibody. However as this experiment was limited to a single repeat, more replicates are also necessary to determine whether IL6 is involved in the activation of TGFβ. Interestingly IL6 siRNA reduced the strain induced response in MMP1 and MMP13, which was similar to that seen in the inhibitory antibody experiments in terms of MMP1. Both of these experiments show that ADAMTS5 and Elastin are not affected by IL6 knockdown. Therefore due to the consistency of results from two different mechanisms of IL6 inhibition, IL6 may be involved in MMP1 regulation. However, more replicates of each of these experiments are needed to validate this data. In addition, confirmation that the IL6 siRNA is effectively abrogating IL6 expression is required as in the current study we were unable to verify knockdown had occurred. However, it follows that if IL6 blockade does regulate TGFβ in SW1353 cells then it is possible that the same crosstalk between IL6 and TGFβ signalling will occur in tenocytes.

The IL6 inhibitory antibody may be inhibiting the luciferase assay, in order to take this work any further this must be confirmed by adding IL6 inhibitory antibody to TGFβ treated cells in the luciferase assay. If this shows that IL6 inhibitory antibody is inhibiting the assay this would indicate that IL6 is not involved in the activation of TGFβ. However because IL6 inhibitory antibody reduces the MMP1 response to mechanical strain in both IL6 inhibitory antibody and siRNA studies that IL6 is regulating the mechanical strain response downstream of TGFβ activation. This may be a result of IL6 controlling localisation of TGFβRI to the early endosome resulting in increased TGFβ signalling or some other means of crosstalk (Zhang, Topley et al. 2005). Chen et al reported that IL6 can regulate TGFβ stimulated apoptosis via PI3K/Akt and STAT (Chen, Chang et al. 1999). However PI3K is not likely to be involved in the IL6 regulation of TGFβ signalling as we have reported that PI3K is not involved in the mechanoregulation of MMP1.

IL6 is a cytokine with potential pro and anti inflammatory activity and is regulated by the NFκB signalling pathway. IL6 signalling involves the IL6R which is made up of two subunits, the IL6R subunit which specifically binds to IL6 and a signal-transducing element also known as gp130. Upon binding of IL6 to the IL6R, gp130 homodimerize, resulting in the activation of associated kinases Janus and tyrosine kinases (JAK), JAK1, JAK2 and TYK2 resulting in the phosphorylation of the cytoplasmic tail of gp130. Signal transducers and activators of transcription (STATs), STAT1 and STAT3 can dock at phosphotyrosine residues on gp130. Upon docking STATs become phosphorylated, dimerize and translocate to the nucleus. At the nucleus they regulate the transcription of target genes (Heinrich, Behrmann et al. 1998; Maggio, Guralnik et al. 2006). Another pathway instigated by the binding of IL6 to the IL6R is the RAS/Raf pathway, activation of which causes the phosphorylation of MAPK and consequent processing of nuclear factor IL6 (NF-IL6). NF-IL6 can also translocate to the nucleus and regulate the transcription of target genes (Maggio, Guralnik et al. 2006). Among other targets of NF-IL6 is the promoter sequence of IL6 and TNF (Akira, Isshiki et al. 1990). IL6 has been shown to suppress the expression of TNF as well as IL1 in mononuclear cells (Schindler, Mancilla et al. 1990), this fits well with our data as we have shown an increase in IL6 mRNA, however we have not been able to detect IL1 or TNF with qRT-PCR. Therefore constitutively expressed or strain stimulated expression of IL6 could be regulating IL1 and TNF, to confirm this further analysis of IL1, IL6 and TNF at the protein level as well as IL6 pathway analysis is required.

Soluble IL6R (sIL6R) can also induce IL6 signalling; IL6 bound IL6R molecules bind to gp130 on the cell surface resulting in activation of gp130 and consequent signalling, this has been termed ‘trans signalling’ (Narazaki, Yasukawa et al. 1993; Maggio, Guralnik et al. 2006). Soluble gp130 however can antagonise IL6 signalling by binding to IL6-sIL6R complexes preventing the signalling through membrane bound gp130 (Jostock, Mullberg et al. 2001; Maggio, Guralnik et al. 2006). As we have preliminary data that shows IL6 inhibition may reduce the level of TGFβ activation and gene regulation in response to mechanical load, to validate this we could use an IL6 receptor fusion protein consisting of IL6R and gp130, which would prevent IL6 signalling (Metz, Wiesinger et al. 2007). The next step would be to characterize the mechanism by which this is controlled. This could be achieved by targeting the IL6 pathway in multiple ways. For example, inhibiting NFκB signalling by specifically inhibiting IKKβ [PS1145 and BMS 345541 (Bain, Plater et al.

2007)] would tell us whether the strain regulated increase in IL6 is mediated via the NFκB pathway. Inhibition of the IL6R using inhibitory antibodies [Tocilizumab (Maggio, Guralnik et al. 2006)], soluble gp130 (Nowell, Richards et al. 2003), or the JAK/STAT and JAK/MAPK pathways by specifically targeting JAK2 [Tyrphostin, also known as AG-490 (Wang, Kirken et al. 1999; Maggio, Guralnik et al. 2006)] we could determine whether IL6 signalling and consequent activation of TGFβ involves the IL6R and JAK/STAT or JAK/MAPK signalling.

siRNA knockdown of all three TGFβ isoforms as well as SMAD4 has shed some light on a complex set of signalling mechanisms. The strain regulation of MMP1 appears to be decreased via the activation of TGFβ1 and despite the involvement of TGFβRI (reported in chapter 4) the canonical TGFβ signalling pathways is not involved, i.e. SMAD4 knockdown does not abrogate this response. MMP13 on the other hand appears to be regulated by means other than TGFβ, as knockdown of TGFβ isoforms does not abrogate the strain induced reduction in MMP13 with strain. Previously we have shown that MMP13 is not regulated via TGFβRI (reported in chapter 5); however SMAD4 appears to be involved in MMP13 regulation with strain as knockdown of SMAD4 abrogates this response. This indicates that some other receptor is involved, perhaps a BMP receptor that also induces SMAD4 translocation to the nucleus. In chapter 4 we showed that ADAMTS5 is stimulated by strain but this does not involve TGFβRI. In the current chapter we have shown that TGFβ1 and TGFβ2 may be involved in the regulation of ADAMTS5, however SMAD4 and TGFβ3 are not. Therefore TGFβ receptors and SMAD proteins are not involved in the regulation of ADAMTS5 in response to mechanical strain. A limitation of the siRNA knockdown studies includes the absence of a scrambled non-strain control; therefore non-strained treated samples cannot be properly compared to a control. To confirm that these different pathways of regulation exist, these experiments must be repeated with the inclusion of all possible control conditions.

siRNA data also indicates that TGFβ3 induces expression of MMP1 and MMP13, as knockdown of TGFβ3 decreases overall expression of both MMP1 and MMP13. This highlights the differential effects of the different TGFβ isoforms. In addition, as TGFβ1 appears to be responsible for the decrease in MMP1 expression in response to mechanical strain and it appears to be the isoform that forms the majority of active TGFβ, TGFβ1 may be the isoform that is responsible for the majority of gene mechanoregulation. This is supported by studies that have characterised the regulation of collagen through TGFβ1 in

response to mechanical strain (Yang, Crawford et al. 2004; Heinemeier, Olesen et al. 2007). However, further study is required to determine the exact role of active TGFβ1 and TGFβ2 isoforms in the regulation of gene expression with mechanical strain.

This chapter describes a set of preliminary data that may lead on to a further understanding of strain mediated modulation of gene expression. Time constraints prevented the completion of three repeats in all these experiments; ideally, to confirm these results at least three repeats would be required. In addition, cell viability assays would be important to confirm that these inhibitors are not inducing apoptosis or necrosis. This could be achieved by collagenase digestion of the gels followed by trypan blue staining and counting of viable and non-viable cells. Confirmation that the inhibitors effectively suppress their targets, the use of more specific inhibitors and the use of multiple inhibitors of the same target that work via different mechanisms would also substantiate the existing data. An increase in the number of genes analysed could lead to a further understanding of a greater breadth of genes that may be regulated by the aforementioned signalling pathways. Further characterisation of the involvement of calcium and IL6 signalling in the regulation of mechanotransduction may be of particular importance. Future studies may also include the inhibition of other pathways such as Rho/Rock and Wnt signalling as these pathways have also been reported in TGFβ signalling crosstalk (Moustakas and Heldin 2005). Rho signalling has already been implicated in the regulation of Tenascin C in response to mechanical loading (Chiquet, Sarasa-Renedo et al. 2004). Therefore confirmation of the involvement of Rho signalling in Tenascin C regulation would be an interesting start point and to determine whether Rho signalling is involved in the regulation of other genes in response to mechanical loading. Interestingly Chiquet and colleagues reported that Tenascin C is regulated by both TGFβ and strain in a similar way via different signalling mechanisms; p38 MAPK is involved in the TGFβ mediated regulation, whereas strain regulation of Tenascin C is mediated via Rho signalling (Chiquet, Sarasa-Renedo et al. 2004). This indicates that Tenascin C and perhaps other genes are not regulated through TGFβ activation and signalling in response to strain. Taken together this data suggests that gene expression in response to mechanical strain involves a complex network of signalling pathways that require further characterisation.

7.5. CONCLUSIONS

1. TGFβ1 and TGFβ3 are the major isoforms activated by mechanical load
2. Neither the strain regulation of TGFβ or gene expression was affected by p38, MKK1, PI3K, COX2, Gap junction, PLC or L and T Ca^{2+} channel inhibition.
3. IL6 inhibition using an antibody reduced strain activation of TGFβ by ~50%.
4. IL6 siRNA did not inhibit the activation of TGFβ; therefore a further mechanism is probably involved in strain mediated activation of TGFβ.
5. Strain regulation of MMP1 was abrogated by the addition of IL6 functionally inhibiting antibody and siRNA knockdown, other genes we measured remained unaffected. This suggests a role for IL6 in MMP1 regulation.
6. MMP1, MMP13 and ADAMTS5 may be regulated by distinct signalling pathways.

CHAPTER 8: Overall Conclusions

Published work has focused on a limited number of genes in response to mechanical load in tendon. In chapter 3 we aimed to document mechanical regulation of a wider range of matrix related genes. Using TLDA analysis we showed that mechanical strain regulates of a wide range of metalloproteinase, TIMPs, cytokines, cell lineage markers and collagens in response to 2-48 hours of 0-5% cyclic loading at 1Hz in tendinopathy derived tenocytes. We showed that the majority of collagens, metalloproteinase and TIMP genes are regulated in an anabolic manner. In addition ADAMTSs, tendon cell lineage 'markers' were increased, and MMPs were decreased after 24 and 48 hours. We showed strain regulation of novel genes which included ADAMTS2, ADAMTS5, ADAMTS10, ADAMTS16 and TIMP3. Gene responses were more robust in cells seeded at the higher cell density, indicating that cell to cell interactions play a role in gene mechanoregulation. These observations led us to focus on gap junctions with respect to calcium signalling (chapter 7) and to integrins in terms of TGFβ activation (chapter 5). We found that not only do tendinopathy derived cells respond to mechanical loading but cells derived from normal and ruptured tendon also show a similar pattern of regulation.

TGFβ is increased in tendinopathy (Fenwick, Curry et al. 2001; Fu, Wang et al. 2002). This inspired our interest in comparing the regulation of genes to strain and TGFβ treatment. In chapter 4 we showed that the mechanical strain regulation of protease and matrix genes at the mRNA level is analogous to those induced by TGFβ stimulation. Furthermore, the inhibition of the TGFβ signalling pathway, using a specific inhibitor of TGFβRI, abrogated the strain-induced changes in mRNA level. This suggested that mechanoregulation of gene expression is mediated via the canonical TGFβ signalling pathway which involves SMAD translocation to the nucleus and consequent transcriptional regulation. Measurement of TGFβ protein expression using a cell based luciferase assay showed that total TGFβ in strained and non-strained samples was similar. However, active TGFβ was significantly increased in strained compared to non-strained controls. TGFβ was

not significantly increased at the mRNA level in response to mechanical load. Therefore application of 5% strain at 1Hz in our model induces TGFβ activation, subsequent signalling and regulation of genes in response to mechanical load. In chapter 6 we used siRNA to inhibit all three of the TGFβ isoforms. We showed that TGFβ1 and TGFβ3 are the major isoforms of TGFβ activated with mechanical strain (approximately 70% and 30% respectively). Although this experiment was only n=1 this data was supported by qRT-PCR where Ct values were of a similar ratio; TGFβ1, TGFβ2 and TGFβ3 had an average Ct of 22.5, 33.3 and 25.6 respectively. Although we have shown that TGFβ1 and TGFβ3 activation occurs in response to mechanical strain, we have not confirmed that both of these isoforms are involved in the regulation of genes seen in response to mechanical strain. Although, siRNA knockdown of TGFβ1 indicates that the anabolic regulation of MMP1 with mechanical strain is induced by TGFβ1, the strain regulation of other genes may be regulated by TGFβ3. Analysis of a broader range of genes in TGFβ1 and TGFβ3 knockdown experiments would indicate whether this was the case.

TGFβ up-regulation at both the protein and mRNA level with mechanical strain has been previously reported in tenocytes (Skutek, van Griensven et al. 2001; Yang, Crawford et al. 2004) and other cell types (Riser, Cortes et al. 1996; Riser, Ladson-Wofford et al. 1999; Kim, Akaike et al. 2002; Baker, Ettenson et al. 2008). TGFβ1 has also been implicated as a regulatory step in the mechanical regulation of collagen (Yang, Crawford et al. 2004; Heinemeier, Olesen et al. 2007), although these studies have not specifically shown that activation of TGFβ is involved. Maeda and colleagues (Maeda, Sakabe et al. 2011) have reported TGFβ activation as a key regulator in scleraxis. However, we are the first to implicate TGFβ activation as a key regulator in the mechanoregulation of metalloproteinases and other matrix genes.

TGFβ activation mechanisms are well characterised in the literature. In chapter 4 we showed that TGFβ activation and not changes in mRNA or protein was responsible for the strain regulated gene expression. We aimed to characterise the method of activation occurring in response to mechanical strain in our system. In chapter 5 we have shown that metallo, serine, cysteine, aspartic proteases and aminopeptidases are not involved in the activation of TGFβ in response to mechanical loading, despite the evidence to show that protease can play an important role in the activation of TGFβ in other systems (Lyons, Keski-Oja et al. 1988; Taipale, Koli et al. 1992; Yu and Stamenkovic 2000; D'Angelo, Billings et al. 2001; Maeda, Dean et al. 2002; Mu, Cambier et al. 2002). TSP1 has also

been reported to be one of the key regulators of TGFβ activation in other systems (Schultz-Cherry and Murphy-Ullrich 1993; Crawford, Stellmach et al. 1998; Ribeiro, Poczatek et al. 1999). In addition there is evidence to suggest that shear force is able to stimulate the activation of TGFβ in blood platelets (Ahamed, Burg et al. 2008), and that activation of TGFβ can be partially facilitated by TSP-1 (Ahamed, Janczak et al. 2009). However, we showed that the interaction between thrombospondin and LAP via the LSKL motif is not involved in the activation of TGFβ in response to mechanical load; this could be due to differences in force (shear and tensional force) or cell type (blood platelets and tenocytes). M6P/IGF-II interaction with LAP has also been shown to regulate TGFβ activation in other tissues (Dennis and Rifkin 1991), here we show that TGFβ activation does not involve the interaction of TGFβ and M6P/IGF-II via M6P. Therefore none of the major mechanisms of TGFβ activation were responsible for the activation of TGFβ in response to strain. A novel and as yet undetermined method of TGFβ activation may be responsible.

As we were unable to pinpoint the mechanism of TGFβ activation induced in response to mechanical loading we widened our studies to encompass a wider range of signalling mediators that may be involved in the mechanical regulation of gene expression we showed in chapter 4. In a range of preliminary experiments we showed that calcium signalling via GAP junctions, phospholipase conversion of PIP_2 to PI_3 kinase, as well as the transport of calcium via L and T type calcium channels are not involved in the activation of TGFβ with strain or proceeding gene regulation. MAP kinase signalling pathways previously thought to be involved in TGFβ signalling crosstalk (P38 MAPK, MKK1 and PI3K kinase signalling pathways) are also not involved in the strain induced activation of TGFβ or consequent gene regulation. And despite the increase in COX2 mRNA expression preceding TGFβ activation COX2 is not involved in the activation of TGFβ or gene regulation in response to mechanical load. IL6 mRNA was also induced at an early time point (2 hours) in response to strain, preceding the activation of TGFβ. Therefore in order to test the hypothesis that IL6 is involved in the activation of TGFβ, we used an IL6 functionally inhibiting antibody against IL6. IL6 abrogation using the inhibitory antibody reduced the activation of TGFβ by ~50%. In contrast, IL6 siRNA knockdown did not show the same abrogation of TGFβ activation. The functionally inhibitory antibody and the IL6 siRNA, however, decreased the MMP1 response to strain. This suggests that IL6 does have some effect upon the regulation of MMP1 in response to

mechanical load. This also showed that different genes are regulated by different means as the other genes analysed did not respond to IL6 inhibition in the same manner.

siRNA knockdown of TGFβ3 indicates that TGFβ3 induces expression of MMP1 and MMP13, as knockdown of TGFβ3 decreases overall expression of both MMP1 and MMP13. This highlights the differential effects of the different TGFβ isoforms. As TGFβ3 increases overall MMP1 and MMP13 expression, it appears that TGFβ1 is the primary TGFβ involved in the regulation of genes in response to mechanical strain. With reference to the anabolic or catabolic affects produced in response to TGFβ1 and TGFβ3 (respectively), differential expression of different isoforms of TGFβ as well as receptor expression could be involved in the development of pathology in tendon (Fenwick, Curry et al. 2001).

siRNA knockdown of the different TGFβ isoforms as well as SMAD4 allowed us to further characterise mechanotransduction pathways. The strain regulation of **MMP1** appears to be decreased via the activation of TGFβ1 and despite the involvement of TGFβRI (reported in chapter 4) the canonical TGFβ signalling pathways is not involved, i.e. SMAD4 knockdown does not abrogate this response. **MMP13** on the other hand appears to be regulated by means other than TGFβ, as knockdown of TGFβ isoforms does not abrogate the strain induced reduction in MMP13 with strain. SMAD4 appears to be involved in MMP13 regulation with strain as knockdown of SMAD4 abrogates this response, although we have also shown that MMP13 is not regulated via TGFβRI (reported in chapter 4). This indicates that some other receptor is involved, perhaps a BMP receptor that also induces SMAD4 translocation to the nucleus. In chapter 5 we showed that **ADAMTS5** is stimulated by strain but this does not involve TGFβRI. In chapter 7 we showed that TGFβ1 and TGFβ2 may be involved in the regulation of ADAMTS5, however SMAD4 and TGFβ3 are not. This suggests that ADAMTS5 is regulated by TGFβ but not as a result of canonical TGFβ signalling.

We therefore have evidence to show that MMP1, MMP13 and ADAMTS5 are regulated via separate pathways, and that each involves TGFβ signalling in some way. However to confirm these different pathways of regulation exist these experiments must be repeated and extended. By understanding this complex set of regulatory mechanisms we may be able to determine whether dysregulation of this system is involved in the development of tendinopathy.

FUTURE WORK

- Repetition of incomplete studies
- Verification of inhibitor efficacy in the studies described in this thesis

IL6 characterisation

- To test whether IL6 antibody is inhibiting the TGFβ luciferase assay
- Validation of the role of IL6 in MMP1 regulation with strain
- Further characterisation of IL6 signalling and how this relates to the strain response

Further characterisation of strain regulated gene expression

- To characterise the different signalling pathways that regulate MMP1, MMP13 and ADAMTS5 in response to mechanical strain
- To determine whether other genes are regulated by the same mechanisms
- To inhibit Rho/Rock and Wnt signalling

TGFβ activation

- Measurement of LTBP and identification of whether LTBP targets the LLC complex to the ECM
- Identification of whether inactive TGFβ measured in the luciferase assay, is associated with a proportion of the LLC
- Identification of TGFβ activation mechanisms (i.e. inhibition of other proteases such as BMP1)

Mechanical stress

- To determine how much strain that the cell seeded collagen gels undergo in the Flexcell^{TM} system, during 48 hours of loading
- To determine the level of strain that tenocytes are exposed to

Abbreviations

α-SMA	Alpha Smooth Muscle Actin
18S	18s Ribosomal Ribonucleic Acid
a1-PI	Alpha 1-Proteinase Inhibitor
a2-M	Alpha 2-Macroglobulin
ACAN	Aggrecan
ACL	Anterior Cruciate Ligament
ACTA1	Alpha Smooth Muscle Actin
ACTA2	α Smooth Muscle Actin
ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin motifs
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride
APP	Amyloid Precursor Protein
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblast Growth Factor
BGN	Biglycan
BMP	Bone Morphogenic Proteins
BSA	Bovine Serum Albumin
CA	Cysteine Array
Ca^{2+}	Calcium
CaCl	Calcium Chloride

cAMP	Cyclic Adenosine Monophosphate
CCD	Charge-Coupled Device
cAMP	Cyclic adenosine monophosphate
cMMP	Chicken MMP;
COL1A1	Collagen, type I, alpha 1
COL2A1	Collagen, type II, alpha 1
COL3A1	Collagen, type III, alpha 1
COL12A1	Collagen, type XII, alpha 1
COL14A1	Collagen, type XIV, alpha 1
COMP	Complex Oligomeric Matrix Protein
COX2	Cyclooxygenase 2
CS	Chondroitin sulphate
CTGF	Connective Tissue Growth Factor
Cx	Connexins
DAG	1,2-diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DCN	Decorin
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP’s	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EEA-1	Early Endosome Antigen-1

ER	Endoplasmic reticulum
ERK	extracellular-signal-regulated kinases
FACIT	Beaded Filament Collagen and Fibril-Associated Collagen with Interrupted Triple Helix
FAK	Focal Adhesion Kinase
Fas-L	Fas Ligand
FBN	Fibronectin
FGF	Fibroblast Growth Factor
Fn	Fibronectin
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF	Growth and Differentiation Factors
GF	Growth Factor;
GPI	Glycosylphosphatidylinositol
GRM	Metabotropic glutamate receptor
GTP	Guanadine Tri-Phosphate
HAPLN1	Link Protein
HB-EGF	Heparin-Binding Epidermal Growth Factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGNC	HUGO Gene Nomenclature Committee
ICAM	Intercellular Adhesion Molecule
IGF	Insulin-like Growth Factor
IGFBP	Insulin-Like Growth Factor Binding Protein
IKK	Inhibitor of Kappa B Kinase
IL	Interleukin
IL1B	Interleukin 1B

IL4	Interleukin 4
IL6	Interleukin 6
IL6R	Interleukin 6 Receptor
IL17A	Interleukin 17A
IL6R	Interleukin-6 Receptor
IP_3	Inositol (1,4,5)-Triphosphate
I-SMAD	Inhibitory SMAD
JNK	c-JUN end terminal Kinases
Kit-L	kit Ligand
KL-1	Kit-Ligand-1
KS	keratin sulphate at one domain
LAP	Latency Associated Peptide
LLC	Large Latent Complex
Ln	Laminin
LTBP	Large TGFβ Binding Protein
LUM	Lumican
M6P	Mannose-6-Phosphate
M6P/IGF-II	Mannose 6-Phosphate/Insulin-Like Growth Factor-II Receptor
MAGP1	Microfibril Associated Glycoproteins
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MCL	Medial Collateral ligament
MCP-3	Monocyte Chemotactic Protein-3
METH-1	Methamphetamine-1 (Human ortholog of ADAMTS-1)
METH-2	Methamphetamine-2

MFS	Marfan syndrome
MKK1	Mitogen Activated protein Kinase Kinase 1
MMP	Matrix Metalloproteinase
MT1-MMP	Membrane-Type Matrix Metalloproteinase
NA	Not Applicable
Na^+	Sodium
NAD^+	Nicotinamide Adenine Dinucleotide
NaOH	Sodium Hydroxide
ND	No Data
NF-IL6	Nuclear Factor Interleukin 6
NFκB	Nuclear Factor Kappa B
NO	Nitric Oxide
NS	Not Significant
NSAID	Non-steroidal Anti-inflammatory Drug
OD	Optimum Density
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PG	Proteoglycan
PGE_2	Prosteglandin E_2
PI_3	Phosphatidylinositol 3
PIP_2	Phophatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
Pro	Proteinase type
pro-IL-1	Pro-Interleukin 1

ProMMP	Latent Matrix Metalloproteinase
proTGF-b	Pro Transforming Growth Factor beta
proTNF-a	Pro Tumor Necrosis Factor-alpha
PTGS2	Cyclooxygenase 2
PUMP	Putative Metalloproteinase
PVDF	Polyvinylidene Flouride membrane
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
RASI-1	Rheumatoid Arthritis Synovium Inflamed 1
RNA	Ribonucleic Acid
ROCK	Rho/ Rho associated protein kinase
ROS	Reactive Oxygen Species
R-SMAD	Receptor Regulated SMAD
SAPK	Stress Activated Protein Kinase
SARA	SMAD anchor for receptor activation
SCX	Scleraxis
SD	Standard Deviation
SDFT	Superficial Digital Flexor Tendon
SDS	Sodium Dodecyl Sylphate
SDS-PAGE	Polyacrylamide Gel Electrophoresis
SE	Standard error
siRNA	Small Interfering Ribonucleic Acid
SLC	Small Latent Complex
SLRP	Small leucine rich proteoglycans
SOX9	SRY(sex determining region Y) -type HMG (High mobility group box)

STAT3	Signal Transducers and Activators of Transcription
TBS	Tris Buffered Saline Solution
TCA	Trichloroacetic Acid
TEMED	Tetramethylethylenediamine
TESPA	3-triethoxysilylpropylamine
TF	Transcription Factor;
TGFB1	Transforming Growth Factor β1
TGFβ	Transforming growth factor beta
TGFβRI	Transforming Growth factor beta receptor I
TGFβRII	Transforming Growth factor beta receptor I
THBS1 / TSP1	Thrombospondin -1
TIMP	Tissue Inhibitor of Metalloproteinase
TLDA	Taqman Low density array
TNC	Tenascin C
TNF	Tumour Necrosis Factor
TNMD	Tenomodulin
TOP1	Topoisomerase 1
TRANCE	TNF-related activation induced cytokine.
TSP1	Thrombospondin-1
uPA	Urokinase Plasminogen Activator
VCAN	Versican
VLA	Very Late Antigen
vWF	Von Willebrand Factor
vWFCP	Von Willebrand Factor-Cleaving Protease
xMMP	Xenopus Matrix Metalloproteinase

Bibliography

Abe, M., N. Oda, et al. (1998). "Cell-associated activation of latent transforming growth factor-beta by calpain." J Cell Physiol **174**(2): 186-193.

Agarwal, S., J. Deschner, et al. (2004). "Role of NF-kappaB transcription factors in antiinflammatory and proinflammatory actions of mechanical signals." Arthritis Rheum **50**(11): 3541-3548.

Ahamed, J., N. Burg, et al. (2008). "In vitro and in vivo evidence for shear-induced activation of latent transforming growth factor-beta1." Blood **112**(9): 3650-3660.

Ahamed, J., C. A. Janczak, et al. (2009). "In vitro and in vivo evidence that thrombospondin-1 (TSP-1) contributes to stirring- and shear-dependent activation of platelet-derived TGF-beta1." PLoS One **4**(8): e6608.

Ahmed, I. M., M. Lagopoulos, et al. (1998). "Blood supply of the Achilles tendon." J Orthop Res **16**(5): 591-596.

Akira, S., H. Isshiki, et al. (1990). "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family." EMBO J **9**(6): 1897-1906.

Alexander, R. M. (2002). "Tendon elasticity and muscle function." Comp Biochem Physiol A Mol Integr Physiol **133**(4): 1001-1011.

Alfredson, H., S. Forsgren, et al. (2001). "In vivo microdialysis and immunohistochemical analyses of tendon tissue demonstrated high amounts of free glutamate and glutamate NMDAR1 receptors, but no signs of inflammation, in Jumper's knee." J Orthop Res **19**(5): 881-886.

Alfredson, H., M. Lorentzon, et al. (2003). "cDNA-arrays and real-time quantitative PCR techniques in the investigation of chronic Achilles tendinosis." J Orthop Res **21**(6): 970-975.

Alfredson, H., K. Thorsen, et al. (1999). "In situ microdialysis in tendon tissue: high levels of glutamate, but not prostaglandin E2 in chronic Achilles tendon pain." Knee Surg Sports Traumatol Arthrosc **7**(6): 378-381.

Ali, M. H., P. T. Mungai, et al. (2006). "Stretch-induced phosphorylation of focal adhesion kinase in endothelial cells: role of mitochondrial oxidants." Am J Physiol Lung Cell Mol Physiol **291**(1): L38-45.

Almekinders, L. C. and J. D. Temple (1998). "Etiology, diagnosis, and treatment of tendonitis: an analysis of the literature." Med Sci Sports Exerc **30**(8): 1183-1190.

Aluwihare, P., Z. Mu, et al. (2009). "Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice." J Cell Sci **122**(Pt 2): 227-232.

Annes, J. P., Y. Chen, et al. (2004). "Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1." J Cell Biol **165**(5): 723-734.

Annes, J. P., J. S. Munger, et al. (2003). "Making sense of latent TGFbeta activation." J Cell Sci **116**(Pt 2): 217-224.

Annes, J. P., D. B. Rifkin, et al. (2002). "The integrin alphaVbeta6 binds and activates latent TGFbeta3." FEBS Lett **511**(1-3): 65-68.

Archambault, J., M. Tsuzaki, et al. (2002). "Stretch and interleukin-1beta induce matrix metalloproteinases in rabbit tendon cells in vitro." J Orthop Res **20**(1): 36-39.

Archambault, J. M., M. K. Elfervig-Wall, et al. (2002). "Rabbit tendon cells produce MMP-3 in response to fluid flow without significant calcium transients." J Biomech **35**(3): 303-309.

Arnoczky, S. P., M. Lavagnino, et al. (2007). "The mechanobiological aetiopathogenesis of tendinopathy: is it the over-stimulation or the under-stimulation of tendon cells?" Int J Exp Pathol **88**(4): 217-226.

Arnoczky, S. P., M. Lavagnino, et al. (2007). "Matrix metalloproteinase inhibitors prevent a decrease in the mechanical properties of stress-deprived tendons: an in vitro experimental study." Am J Sports Med **35**(5): 763-769.

Arnoczky, S. P., M. Lavagnino, et al. (2008). "Loss of Homeostatic Strain Alters Mechanostat "Set Point" of Tendon Cells In Vitro." Clin Orthop Relat Res.

Arnoczky, S. P., M. Lavagnino, et al. (2002). "In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy." J Orthop Res **20**(1): 29-35.

Arnoczky, S. P., T. Tian, et al. (2004). "Ex vivo static tensile loading inhibits MMP-1 expression in rat tail tendon cells through a cytoskeletally based mechanotransduction mechanism." J Orthop Res **22**(2): 328-333.

Arnoczky, S. P., T. Tian, et al. (2002). "Activation of stress-activated protein kinases (SAPK) in tendon cells following cyclic strain: the effects of strain frequency, strain magnitude, and cytosolic calcium." J Orthop Res **20**(5): 947-952.

Asano, Y., H. Ihn, et al. (2005). "Increased expression of integrin alpha(v)beta3 contributes to the establishment of autocrine TGF-beta signaling in scleroderma fibroblasts." J Immunol **175**(11): 7708-7718.

Asano, Y., H. Ihn, et al. (2005). "Involvement of alphavbeta5 integrin-mediated activation of latent transforming growth factor beta1 in autocrine transforming growth factor beta signaling in systemic sclerosis fibroblasts." Arthritis Rheum **52**(9): 2897-2905.

Ashida, N., H. Takechi, et al. (2003). "Vortex-mediated mechanical stress induces integrin-dependent cell adhesion mediated by inositol 1,4,5-trisphosphate-sensitive Ca2+ release in THP-1 cells." J Biol Chem **278**(11): 9327-9331.

Astrom, M. and A. Rausing (1995). "Chronic Achilles tendinopathy. A survey of surgical and histopathologic findings." Clin Orthop Relat Res(316): 151-164.

Asundi, K. R. and D. M. Rempel (2008). "Cyclic loading inhibits expression of MMP-3 but not MMP-1 in an in vitro rabbit flexor tendon model." Clin Biomech (Bristol, Avon) **23**(1): 117-121.

Asundi, K. R. and D. M. Rempel (2008). "MMP-1, IL-1beta, and COX-2 mRNA expression is modulated by static load in rabbit flexor tendons." Ann Biomed Eng **36**(2): 237-243.

Atfi, A., E. Dumont, et al. (2007). "The disintegrin and metalloproteinase ADAM12 contributes to TGF-beta signaling through interaction with the type II receptor." J Cell Biol **178**(2): 201-208.

Bader, B. L., H. Rayburn, et al. (1998). "Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins." Cell **95**(4): 507-519.

Badley, E. M. and A. Tennant (1993). "Impact of disablement due to rheumatic disorders in a British population: estimates of severity and prevalence from the Calderdale Rheumatic Disablement Survey." Ann Rheum Dis **52**(1): 6-13.

Bain, J., L. Plater, et al. (2007). "The selectivity of protein kinase inhibitors: a further update." Biochem J **408**(3): 297-315.

Baker, A. B., D. S. Ettenson, et al. (2008). "Endothelial cells provide feedback control for vascular remodeling through a mechanosensitive autocrine TGF-beta signaling pathway." Circ Res **103**(3): 289-297.

Banes, A. J., M. Tsuzaki, et al. (1995). "Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals." Biochem Cell Biol **73**(7-8): 349-365.

Banes, A. J., P. Weinhold, et al. (1999). "Gap junctions regulate responses of tendon cells ex vivo to mechanical loading." Clin Orthop Relat Res(367 Suppl): S356-370.

Barcellos-Hoff, M. H., R. Derynck, et al. (1994). "Transforming growth factor-beta activation in irradiated murine mammary gland." J Clin Invest **93**(2): 892-899.

Barcellos-Hoff, M. H. and T. A. Dix (1996). "Redox-mediated activation of latent transforming growth factor-beta 1." Mol Endocrinol **10**(9): 1077-1083.

Bax, D. V., S. E. Bernard, et al. (2003). "Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by alpha 5 beta 1 and alpha v beta 3 integrins." J Biol Chem **278**(36): 34605-34616.

Birch, H. L. (2007). "Tendon matrix composition and turnover in relation to functional requirements." Int J Exp Pathol **88**(4): 241-248.

Birch, H. L., J. V. Bailey, et al. (1999). "Age-related changes to the molecular and cellular components of equine flexor tendons." Equine Vet J **31**(5): 391-396.

Bode, W. (2003). "Structural basis of matrix metalloproteinase function." Biochem Soc Symp(70): 1-14.

Boitano, S., E. R. Dirksen, et al. (1992). "Intercellular propagation of calcium waves mediated by inositol trisphosphate." Science **258**(5080): 292-295.

Booth, A. J., K. Csencsits-Smith, et al. (2010). "Connective tissue growth factor promotes fibrosis downstream of TGFbeta and IL-6 in chronic cardiac allograft rejection." Am J Transplant **10**(2): 220-230.

Borkakoti, N. (1998). "Matrix metalloproteases: variations on a theme." Prog Biophys Mol Biol **70**(1): 73-94.

Brancaccio, M., E. Hirsch, et al. (2006). "Integrin signalling: the tug-of-war in heart hypertrophy." Cardiovasc Res **70**(3): 422-433.

Brooks, P. C., S. Stromblad, et al. (1996). "Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3." Cell **85**(5): 683-693.

Buckwalter, J. A. (1996). "Effects of early motion on healing of musculoskeletal tissues." Hand Clin **12**(1): 13-24.

Buttice, G., M. Duterque-Coquillaud, et al. (1996). "Erg, an Ets-family member, differentially regulates human collagenase1 (MMP1) and stromelysin1 (MMP3) gene expression by physically interacting with the Fos/Jun complex." Oncogene **13**(11): 2297-2306.

Callahan, J. F., J. L. Burgess, et al. (2002). "Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5)." J Med Chem **45**(5): 999-1001.

Canty, E. G. and K. E. Kadler (2005). "Procollagen trafficking, processing and fibrillogenesis." J Cell Sci **118**(Pt 7): 1341-1353.

Cao, L., M. T. Young, et al. (2007). "Thr339-to-serine substitution in rat P2X2 receptor second transmembrane domain causes constitutive opening and indicates a gating role for Lys308." J Neurosci **27**(47): 12916-12923.

Chakravarti, S., T. Magnuson, et al. (1998). "Lumican regulates collagen fibril assembly: skin fragility and corneal opacity in the absence of lumican." J Cell Biol **141**(5): 1277-1286.

Chard, M. D., T. E. Cawston, et al. (1994). "Rotator cuff degeneration and lateral epicondylitis: a comparative histological study." Ann Rheum Dis **53**(1): 30-34.

Chaudhry, S. S., S. A. Cain, et al. (2007). "Fibrillin-1 regulates the bioavailability of TGFbeta1." J Cell Biol **176**(3): 355-367.

Chen, R. H., M. C. Chang, et al. (1999). "Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways." J Biol Chem **274**(33): 23013-23019.

Chiquet, C., M. A. Custaud, et al. (2003). "Changes in intraocular pressure during prolonged (7-day) head-down tilt bedrest." J Glaucoma **12**(3): 204-208.

Chiquet, M. (1999). "Regulation of extracellular matrix gene expression by mechanical stress." Matrix Biol **18**(5): 417-426.

Chiquet, M., A. Sarasa-Renedo, et al. (2004). "Induction of tenascin-C by cyclic tensile strain versus growth factors: distinct contributions by Rho/ROCK and MAPK signaling pathways." Biochim Biophys Acta **1693**(3): 193-204.

Choi, Y., E. Kim, et al. (2010). "Site-specific inhibition of integrin alpha v beta 3-vitronectin association by a ser-asp-val sequence through an Arg-Gly-Asp-binding site of the integrin." Proteomics **10**(1): 72-80.

Clark, I. M., D. A. Young, et al. (2010). Matrix metalloproteinase protocols. [Totowa, N.J.], Humana.

Clegg, P. D., S. Strassburg, et al. (2007). "Cell phenotypic variation in normal and damaged tendons." Int J Exp Pathol **88**(4): 227-235.

Colige, A., A. Beschin, et al. (1995). "Characterization and partial amino acid sequencing of a 107-kDa procollagen I N-proteinase purified by affinity chromatography on immobilized type XIV collagen." J Biol Chem **270**(28): 16724-16730.

Colige, A., S. W. Li, et al. (1997). "cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components." Proc Natl Acad Sci U S A **94**(6): 2374-2379.

Corps, A. N., G. C. Jones, et al. (2008). "The regulation of aggrecanase ADAMTS-4 expression in human Achilles tendon and tendon-derived cells." Matrix Biol **27**(5): 393-401.

Corps, A. N., A. H. Robinson, et al. (2012). "Changes in matrix protein biochemistry and the expression of mRNA encoding matrix proteins and metalloproteinases in posterior tibialis tendinopathy." Ann Rheum Dis.

Corps, A. N., A. H. Robinson, et al. (2006). "Increased expression of aggrecan and biglycan mRNA in Achilles tendinopathy." Rheumatology (Oxford) **45**(3): 291-294.

Corps, A. N., A. H. Robinson, et al. (2004). "Versican splice variant messenger RNA expression in normal human Achilles tendon and tendinopathies." Rheumatology (Oxford) **43**(8): 969-972.

Craig, D. H., B. Haimovich, et al. (2007). "Alpha-actinin-1 phosphorylation modulates pressure-induced colon cancer cell adhesion through regulation of focal adhesion kinase-Src interaction." Am J Physiol Cell Physiol **293**(6): C1862-1874.

Crawford, S. E., V. Stellmach, et al. (1998). "Thrombospondin-1 is a major activator of TGF-beta1 in vivo." Cell **93**(7): 1159-1170.

Cross, N. A., S. Chandrasekharan, et al. (2005). "The expression and regulation of ADAMTS-1, -4, -5, -9, and -15, and TIMP-3 by TGFbeta1 in prostate cells: relevance to the accumulation of versican." Prostate **63**(3): 269-275.

D'Angelo, M., P. C. Billings, et al. (2001). "Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta." J Biol Chem **276**(14): 11347-11353.

Dai, P., T. Nakagami, et al. (2007). "Cx43 mediates TGF-beta signaling through competitive Smads binding to microtubules." Mol Biol Cell **18**(6): 2264-2273.

Danielson, K. G., H. Baribault, et al. (1997). "Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility." J Cell Biol **136**(3): 729-743.

de Mos, M., B. van El, et al. (2007). "Achilles tendinosis: changes in biochemical composition and collagen turnover rate." Am J Sports Med **35**(9): 1549-1556.

Dennis, P. A. and D. B. Rifkin (1991). "Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor." Proc Natl Acad Sci U S A **88**(2): 580-584.

Determe, D., M. Rongieres, et al. (1996). "Anatomic study of the tendinous rotator cuff of the shoulder." Surg Radiol Anat **18**(3): 195-200.

DiCesare, P., N. Hauser, et al. (1994). "Cartilage oligomeric matrix protein (COMP) is an abundant component of tendon." FEBS Lett **354**(2): 237-240.

Dickson, M. C., J. S. Martin, et al. (1995). "Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice." Development **121**(6): 1845-1854.

Docheva, D., E. B. Hunziker, et al. (2005). "Tenomodulin is necessary for tenocyte proliferation and tendon maturation." Mol Cell Biol **25**(2): 699-705.

Drummond, A. H., P. Beckett, et al. (1999). "Preclinical and clinical studies of MMP inhibitors in cancer." Ann N Y Acad Sci **878**: 228-235.

Duance, V. C., D. J. Restall, et al. (1977). "The location of three collagen types in skeletal muscle." FEBS Lett **79**(2): 248-252.

Dubois, C. M., F. Blanchette, et al. (2001). "Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme." Am J Pathol **158**(1): 305-316.

Dudhia, J., C. M. Scott, et al. (2007). "Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity." Aging Cell **6**(4): 547-556.

Duncan, M. R., K. S. Frazier, et al. (1999). "Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP." FASEB J **13**(13): 1774-1786.

Edwards, D. R., M. M. Handsley, et al. (2008). "The ADAM metalloproteinases." Mol Aspects Med **29**(5): 258-289.

Egerbacher, M., S. P. Arnoczky, et al. (2008). "Loss of homeostatic tension induces apoptosis in tendon cells: an in vitro study." Clin Orthop Relat Res **466**(7): 1562-1568.

Egerbacher, M., O. Caballero, et al. (2007). "Loss of homeostatic tension induces up-regulation of connexin-43 in tendon cells." In: Transactions of the Orthopaedic research society; Orthopaedic Research Society, 2007.

Elfervig, M., Archambault, J., Herzog, W., Bynum, D., Banes, A.J. (2001). "Mechanical stretching induces increased intracellular Ca^{2+} in human tendon cells [Abstract]." Transactions of the 47th Annual meeting of Orthopeodic Research Society(26): 566.

Elfervig, M., E. Francke, et al. (2000). "Fluid-induced shear stress activates human tendon cells to signal through multiple calcium dependant pathways." In: Transactions of

the Orthopaedic research society; Orlando, Florida, Orthopaedic Research Society, 2000.

Elfervig, M., M. Lotano, et al. (2001). "Fluid-induced shear stress modulates Cx-43 expression in avian tendon cells but does not induce a calcium signal." In: Transactions of the Orthopaedic research society; San Francisco, California, Orthopaedic Research Society, 2001.

Espira, L., L. Lamoureux, et al. (2009). "The basic helix-loop-helix transcription factor scleraxis regulates fibroblast collagen synthesis." J Mol Cell Cardiol **47**(2): 188-195.

Fedorczyk, J. M. (2006). "Tennis elbow: blending basic science with clinical practice." J Hand Ther **19**(2): 146-153.

Fenwick, S. A., V. Curry, et al. (2001). "Expression of transforming growth factor-beta isoforms and their receptors in chronic tendinosis." J Anat **199**(Pt 3): 231-240.

Fenwick, S. A., B. L. Hazleman, et al. (2002). "The vasculature and its role in the damaged and healing tendon." Arthritis Res **4**(4): 252-260.

Fernandes, R. J., S. Hirohata, et al. (2001). "Procollagen II amino propeptide processing by ADAMTS-3. Insights on dermatosparaxis." J Biol Chem **276**(34): 31502-31509.

Fox, A. M., B. F. Jones, et al. (2005). "Reduced load response in tenocytes from P2Y1/P2Y2 purinoceptor null mice." In: Transactions of the Orthopaedic research society; Orthopaedic Research Society, 2005.

Franke, E., A. J. Banes, et al. (2000). "Fluid-induced shear stress increases [Ca2+]ie in cultured human tendon epitenon cells." In: Transactions of the Orthopaedic research society; Orlando, Florida, Orthopaedic Research Society, 2000.

Frey, C., M. Shereff, et al. (1990). "Vascularity of the posterior tibial tendon." J Bone Joint Surg Am **72**(6): 884-888.

Frost, H. M. (1987). "Bone "mass" and the "mechanostat": a proposal." Anat Rec **219**(1): 1-9.

Fu, S. C., B. P. Chan, et al. (2002). "Increased expression of matrix metalloproteinase 1 (MMP1) in 11 patients with patellar tendinosis." Acta Orthop Scand **73**(6): 658-662.

Fu, S. C., W. Wang, et al. (2002). "Increased expression of transforming growth factor-beta1 in patellar tendinosis." Clin Orthop Relat Res(400): 174-183.

Funderburgh, J. L., B. Caterson, et al. (1987). "Distribution of proteoglycans antigenically related to corneal keratan sulfate proteoglycan." J Biol Chem **262**(24): 11634-11640.

Gardner, K., S. P. Arnoczky, et al. (2008). "The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: An in vitro experimental study." Disabil Rehabil: 1-7.

Gardner, K., M. Lavagnino, et al. (2012). "Re-establishment of cytoskeletal tensional homeostasis in lax tendons occurs through an actin-mediated cellular contraction of the extracellular matrix." J Orthop Res.

Garvin, J., J. Qi, et al. (2003). "Novel system for engineering bioartificial tendons and application of mechanical load." Tissue Eng **9**(5): 967-979.

Ge, G. and D. S. Greenspan (2006). "BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein." J Cell Biol **175**(1): 111-120.

Gelberman, R. H. (1985). "Flexor tendon physiology: tendon nutrition and cellular activity in injury and repair." Instr Course Lect **34**: 351-360.

Gelse, K., E. Poschl, et al. (2003). "Collagens--structure, function, and biosynthesis." Adv Drug Deliv Rev **55**(12): 1531-1546.

Gendron, C., M. Kashiwagi, et al. (2007). "Proteolytic activities of human ADAMTS-5: comparative studies with ADAMTS-4." J Biol Chem **282**(25): 18294-18306.

Gentry, L. E. and B. W. Nash (1990). "The pro domain of pre-pro-transforming growth factor beta 1 when independently expressed is a functional binding protein for the mature growth factor." Biochemistry **29**(29): 6851-6857.

Gever, J. R., D. A. Cockayne, et al. (2006). "Pharmacology of P2X channels." Pflugers Arch **452**(5): 513-537.

Gilbert, H. T., J. A. Hoyland, et al. (2011). "The involvement of interleukin-1 and interleukin-4 in the response of human annulus fibrosus cells to cyclic tensile strain: an altered mechanotransduction pathway with degeneration." Arthritis Res Ther **13**(1): R8.

Gillery, P., A. Leperre, et al. (1992). "Insulin-like growth factor-I (IGF-I) stimulates protein synthesis and collagen gene expression in monolayer and lattice cultures of fibroblasts." J Cell Physiol **152**(2): 389-396.

Gilpin, B. J., F. Loechel, et al. (1998). "A novel, secreted form of human ADAM 12 (meltrin alpha) provokes myogenesis in vivo." J Biol Chem **273**(1): 157-166.

Gleizes, P. E., R. C. Beavis, et al. (1996). "Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1." J Biol Chem **271**(47): 29891-29896.

Gomez-Gaviro, M., M. Dominguez-Luis, et al. (2007). "Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding." J Immunol **178**(12): 8053-8063.

Goodison, S., V. Urquidi, et al. (1999). "CD44 cell adhesion molecules." Mol Pathol **52**(4): 189-196.

Graff, R. D., E. R. Lazarowski, et al. (2000). "ATP release by mechanically loaded porcine chondrons in pellet culture." Arthritis Rheum **43**(7): 1571-1579.

Grotendorst, G. R. (1997). "Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts." Cytokine Growth Factor Rev **8**(3): 171-179.

Gupta, V. and K. J. Grande-Allen (2006). "Effects of static and cyclic loading in regulating extracellular matrix synthesis by cardiovascular cells." Cardiovasc Res **72**(3): 375-383.

Hakkinen, L., S. Strassburger, et al. (2000). "A role for decorin in the structural organization of periodontal ligament." Lab Invest **80**(12): 1869-1880.

Halasz, K., A. Kassner, et al. (2007). "COMP acts as a catalyst in collagen fibrillogenesis." J Biol Chem **282**(43): 31166-31173.

Hamada, K., N. Takuwa, et al. (1998). "Stretch activates Jun N-terminal kinase/stress-activated protein kinase in vascular smooth muscle cells through mechanisms involving autocrine ATP stimulation of purinoceptors." J Biol Chem **273**(11): 6334-6340.

Hardingham, T. E. and A. J. Fosang (1992). "Proteoglycans: many forms and many functions." FASEB J **6**(3): 861-870.

Hattori, N., D. A. Carrino, et al. (2011). "Pericellular versican regulates the fibroblast-myofibroblast transition: a role for ADAMTS5 protease-mediated proteolysis." J Biol Chem **286**(39): 34298-34310.

Hattori, T., S. Matsunaga, et al. (2006). "Inhibition of nifedipine-induced proliferation of cultured human gingival fibroblasts by Saiko, a Chinese herbal medicine." Phytother Res **20**(8): 704-707.

Hedbom, E., P. Antonsson, et al. (1992). "Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage." J Biol Chem **267**(9): 6132-6136.

Hedstrom, L. (2002). "Serine protease mechanism and specificity." Chem Rev **102**(12): 4501-4524.

Heinemeier, K. M., J. L. Olesen, et al. (2007). "Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types." J Physiol **582**(Pt 3): 1303-1316.

Heinemeier, K. M., J. L. Olesen, et al. (2009). "Effect of unloading followed by reloading on expression of collagen and related growth factors in rat tendon and muscle." J Appl Physiol **106**(1): 178-186.

Heinrich, P. C., I. Behrmann, et al. (1998). "Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway." Biochem J **334 (Pt 2)**: 297-314.

Herchenhan, A., N. S. Kalson, et al. (2012). "Tenocyte contraction induces crimp formation in tendon-like tissue." Biomech Model Mechanobiol **11**(3-4): 449-459.

Hildebrand, A., M. Romaris, et al. (1994). "Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta." Biochem J **302 (Pt 2)**: 527-534.

Hinz, B., S. H. Phan, et al. (2007). "The myofibroblast: one function, multiple origins." Am J Pathol **170**(6): 1807-1816.

Hung, C. T., F. D. Allen, et al. (1997). "Intracellular calcium response of ACL and MCL ligament fibroblasts to fluid-induced shear stress." Cell Signal **9**(8): 587-594.

Hyc, A., A. Osiecka-Iwan, et al. (2011). "Influence of LPS, TNF, TGF-ss1 and IL-4 on the expression of MMPs, TIMPs and selected cytokines in rat synovial membranes incubated in vitro." Int J Mol Med **27**(1): 127-137.

Hynes, R. O. (1992). "Integrins: versatility, modulation, and signaling in cell adhesion." Cell **69**(1): 11-25.

Ignotz, R. A. and J. Massague (1986). "Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix." J Biol Chem **261**(9): 4337-4345.

Ingber, D. E. (1997). "Tensegrity: the architectural basis of cellular mechanotransduction." Annu Rev Physiol **59**: 575-599.

Inman, G. J., F. J. Nicolas, et al. (2002). "SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7." Mol Pharmacol **62**(1): 65-74.

Ippolito, E., P. G. Natali, et al. (1980). "Morphological, immunochemical, and biochemical study of rabbit achilles tendon at various ages." J Bone Joint Surg Am **62**(4): 583-598.

Ireland, D., R. Harrall, et al. (2001). "Multiple changes in gene expression in chronic human Achilles tendinopathy." Matrix Biol **20**(3): 159-169.

Jelinsky, S. A., J. Archambault, et al. (2010). "Tendon-selective genes identified from rat and human musculoskeletal tissues." J Orthop Res **28**(3): 289-297.

Jelinsky, S. A., S. A. Rodeo, et al. (2011). "Regulation of gene expression in human tendinopathy." BMC Musculoskelet Disord **12**: 86.

Jones, B. F., M. E. Wall, et al. (2005). "Ligament cells stretch-adapted on a microgrooved substrate increase intercellular communication in response to a mechanical stimulus." J Biomech **38**(8): 1653-1664.

Jones, B. F., X. Yang, et al. (2005). "P2Y1/P2Y2-Null tendons exhibit a decreased intracellular calcium response to uniaxial strain and ATP." In: Transactions of the Orthopaedic research society; Orthopaedic Research Society, 2005.

Jones, G. C., A. N. Corps, et al. (2006). "Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon." Arthritis Rheum **54**(3): 832-842.

Jonk, L. J., S. Itoh, et al. (1998). "Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer." J Biol Chem **273**(33): 21145-21152.

Jostock, T., J. Mullberg, et al. (2001). "Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses." Eur J Biochem **268**(1): 160-167.

Kaartinen, V., J. W. Voncken, et al. (1995). "Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction." Nat Genet **11**(4): 415-421.

Kadler, K. E., C. Baldock, et al. (2007). "Collagens at a glance." J Cell Sci **120**(Pt 12): 1955-1958.

Kadler, K. E., A. Hill, et al. (2008). "Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators." Curr Opin Cell Biol **20**(5): 495-501.

Kadler, K. E., D. F. Holmes, et al. (1996). "Collagen fibril formation." Biochem J **316 (Pt 1)**: 1-11.

Kamijo, M., T. Haraguchi, et al. (2006). "The function of connexin 43 on the differentiation of rat bone marrow cells in culture." Biomed Res **27**(6): 289-295.

Kannus, P. and L. Jozsa (1991). "Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients." J Bone Joint Surg Am **73**(10): 1507-1525.

Kar, R., N. Batra, et al. (2012). "Biological role of connexin intercellular channels and hemichannels." Arch Biochem Biophys **524**(1): 2-15.

Karousou, E., M. Ronga, et al. (2008). "Collagens, proteoglycans, MMP-2, MMP-9 and TIMPs in human achilles tendon rupture." Clin Orthop Relat Res **466**(7): 1577-1582.

Kashiwagi, M., M. Tortorella, et al. (2001). "TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5)." J Biol Chem **276**(16): 12501-12504.

Kastelic, J., A. Galeski, et al. (1978). "The multicomposite structure of tendon." Connect Tissue Res **6**(1): 11-23.

Katsumi, A., T. Naoe, et al. (2005). "Integrin activation and matrix binding mediate cellular responses to mechanical stretch." J Biol Chem **280**(17): 16546-16549.

Kawabata, H., T. Katsura, et al. (2009). "Stress deprivation from the patellar tendon induces apoptosis of fibroblasts in vivo with activation of mitogen-activated protein kinases." J Biomech **42**(15): 2611-2615.

Khan, K. M., J. L. Cook, et al. (1999). "Histopathology of common tendinopathies. Update and implications for clinical management." Sports Med **27**(6): 393-408.

Khan, K. M., J. L. Cook, et al. (2000). "Overuse tendinosis, not tendinitis. Part 1: A new paradigm for a difficult clinical problem." The physician and sportsmedicine Retrieved 5, 28.

Kim, S. G., T. Akaike, et al. (2002). "Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells." Cell Struct Funct **27**(3): 139-144.

Kjaer, M., H. Langberg, et al. (2009). "From mechanical loading to collagen synthesis, structural changes and function in human tendon." Scand J Med Sci Sports **19**(4): 500-510.

Kondou, H., S. Mushiake, et al. (2003). "A blocking peptide for transforming growth factor-beta1 activation prevents hepatic fibrosis in vivo." J Hepatol **39**(5): 742-748.

Koskinen, S. O., K. M. Heinemeier, et al. (2004). "Physical exercise can influence local levels of matrix metalloproteinases and their inhibitors in tendon-related connective tissue." J Appl Physiol **96**(3): 861-864.

Kuo, C. K., B. C. Petersen, et al. (2008). "Spatiotemporal protein distribution of TGF-betas, their receptors, and extracellular matrix molecules during embryonic tendon development." Dev Dyn **237**(5): 1477-1489.

Kveiborg, M., R. Albrechtsen, et al. (2008). "Cellular roles of ADAM12 in health and disease." Int J Biochem Cell Biol **40**(9): 1685-1702.

Lambert, A., B. V. Nusgens, et al. (1998). "Mechano-sensing and mechano-reaction of soft connective tissue cells." Adv Space Res **21**(8-9): 1081-1091.

Lambert, C. A., A. C. Colige, et al. (2001). "Distinct pathways in the over-expression of matrix metalloproteinases in human fibroblasts by relaxation of mechanical tension." Matrix Biol **20**(7): 397-408.

Lambert, C. A., C. M. Lapiere, et al. (1998). "An interleukin-1 loop is induced in human skin fibroblasts upon stress relaxation in a three-dimensional collagen gel but is not involved in the up-regulation of matrix metalloproteinase 1." J Biol Chem **273**(36): 23143-23149.

Langberg, H., J. L. Olesen, et al. (2002). "Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans." J Physiol **542**(Pt 3): 985-990.

Langholz, O., D. Rockel, et al. (1995). "Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins." J Cell Biol **131**(6 Pt 2): 1903-1915.

Laurent, G. J. (1987). "Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass." Am J Physiol **252**(1 Pt 1): C1-9.

Lavagnino, M. and S. P. Arnoczky (2005). "In vitro alterations in cytoskeletal tensional homeostasis control gene expression in tendon cells." J Orthop Res **23**(5): 1211-1218.

Lavagnino, M., S. P. Arnoczky, et al. (2006). "Isolated fibrillar damage in tendons stimulates local collagenase mRNA expression and protein synthesis." J Biomech **39**(13): 2355-2362.

Lavagnino, M., S. P. Arnoczky, et al. (2003). "Effect of amplitude and frequency of cyclic tensile strain on the inhibition of MMP-1 mRNA expression in tendon cells: an in vitro study." Connect Tissue Res **44**(3-4): 181-187.

Law, G. J., J. A. Pachter, et al. (1990). "Thapsigargin, but not caffeine, blocks the ability of thyrotropin-releasing hormone to release Ca2+ from an intracellular store in GH4C1 pituitary cells." Biochem J **267**(2): 359-364.

Le Goff, C., F. Morice-Picard, et al. (2008). "ADAMTSL2 mutations in geleophysic dysplasia demonstrate a role for ADAMTS-like proteins in TGF-beta bioavailability regulation." Nat Genet **40**(9): 1119-1123.

Le Pabic, H., D. Bonnier, et al. (2003). "ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling." Hepatology **37**(5): 1056-1066.

Lee, N. V., J. C. Rodriguez-Manzaneque, et al. (2005). "Fibulin-1 acts as a cofactor for the matrix metalloprotease ADAMTS-1." J Biol Chem **280**(41): 34796-34804.

Legerlotz, K., E. R. Jones, et al. (2012). "Increased expression of IL-6 family members in tendon pathology." Rheumatology (Oxford).

Legerlotz, K., G. C. Jones, et al. (2011). "Cyclic loading of tendon fascicles using a novel fatigue loading system increases interleukin-6 expression by tenocytes." Scand J Med Sci Sports.

Legerlotz, K., P. Schjerling, et al. (2007). "The effect of running, strength, and vibration strength training on the mechanical, morphological, and biochemical properties of the Achilles tendon in rats." J Appl Physiol **102**(2): 564-572.

Leigh, D. R., E. L. Abreu, et al. (2008). "Changes in gene expression of individual matrix metalloproteinases differ in response to mechanical unloading of tendon fascicles in explant culture." J Orthop Res **26**(10): 1306-1312.

Lejard, V., G. Brideau, et al. (2007). "Scleraxis and NFATc regulate the expression of the pro-alpha1(I) collagen gene in tendon fibroblasts." J Biol Chem **282**(24): 17665-17675.

Li, J. H., F. H. Chen, et al. (2008). "[Study on inhibitory effects of matrix metalloproteinase inhibitor on migration of cultured human lens epithelial cells]." Zhonghua Yan Ke Za Zhi **44**(4): 315-320.

Li, S. W., A. L. Sieron, et al. (1996). "The C-proteinase that processes procollagens to fibrillar collagens is identical to the protein previously identified as bone morphogenic protein-1." Proc Natl Acad Sci U S A **93**(10): 5127-5130.

Lian, O., A. Scott, et al. (2007). "Excessive apoptosis in patellar tendinopathy in athletes." Am J Sports Med **35**(4): 605-611.

Lo, I. K., L. L. Marchuk, et al. (2004). "Matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase mRNA levels are specifically altered in torn rotator cuff tendons." Am J Sports Med **32**(5): 1223-1229.

Lockwood, C. J., P. Toti, et al. (2005). "Mechanisms of abruption-induced premature rupture of the fetal membranes: thrombin-enhanced interleukin-8 expression in term decidua." Am J Pathol **167**(5): 1443-1449.

Loechel, F., J. W. Fox, et al. (2000). "ADAM 12-S cleaves IGFBP-3 and IGFBP-5 and is inhibited by TIMP-3." Biochem Biophys Res Commun **278**(3): 511-515.

Loechel, F., B. J. Gilpin, et al. (1998). "Human ADAM 12 (meltrin alpha) is an active metalloprotease." J Biol Chem **273**(27): 16993-16997.

Lu, M., J. S. Munger, et al. (2002). "Integrin alpha8beta1 mediates adhesion to LAP-TGFbeta1." J Cell Sci **115**(Pt 23): 4641-4648.

Lyons, R. M., L. E. Gentry, et al. (1990). "Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin." J Cell Biol **110**(4): 1361-1367.

Lyons, R. M., J. Keski-Oja, et al. (1988). "Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium." J Cell Biol **106**(5): 1659-1665.

Maeda, E., C. Fleischmann, et al. (2010). "Functional analysis of tenocytes gene expression in tendon fascicles subjected to cyclic tensile strain." Connect Tissue Res **51**(6): 434-444.

Maeda, E., J. C. Shelton, et al. (2009). "Differential regulation of gene expression in isolated tendon fascicles exposed to cyclic tensile strain in vitro." J Appl Physiol **106**(2): 506-512.

Maeda, S., D. D. Dean, et al. (2002). "The first stage of transforming growth factor beta1 activation is release of the large latent complex from the extracellular matrix of growth plate chondrocytes by matrix vesicle stromelysin-1 (MMP-3)." Calcif Tissue Int **70**(1): 54-65.

Maeda, T., T. Sakabe, et al. (2011). "Conversion of Mechanical Force into TGF-beta-Mediated Biochemical Signals." Curr Biol **21**(11): 933-941.

Maggio, M., J. M. Guralnik, et al. (2006). "Interleukin-6 in aging and chronic disease: a magnificent pathway." J Gerontol A Biol Sci Med Sci **61**(6): 575-584.

Majerus, E. M., X. Zheng, et al. (2003). "Cleavage of the ADAMTS13 propeptide is not required for protease activity." J Biol Chem **278**(47): 46643-46648.

Martin, J. A., D. Mehr, et al. (2003). "The role of tenascin-C in adaptation of tendons to compressive loading." Biorheology **40**(1-3): 321-329.

Massam-Wu, T., M. Chiu, et al. (2010). "Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF beta." J Cell Sci **123**(Pt 17): 3006-3018.

Matsumoto, N., E. Ishimura, et al. (2003). "Blocking of alpha 5 integrin stimulates production of TGF-beta and PAI-1 by human mesangial cells." Biochem Biophys Res Commun **305**(4): 815-819.

McCormick, A., J. Charlton, et al. (1995). "Assessing health needs in primary care. Morbidity study from general practice provides another source of information." BMJ **310**(6993): 1534.

McNeilly, C. M., A. J. Banes, et al. (1996). "Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions." J Anat **189 (Pt 3)**: 593-600.

Meek, R. L., S. K. Cooney, et al. (2003). "Amino acids induce indicators of response to injury in glomerular mesangial cells." Am J Physiol Renal Physiol **285**(1): F79-86.

Meng, X., K. Mavromatis, et al. (1999). "Mechanical stretching of human saphenous vein grafts induces expression and activation of matrix-degrading enzymes associated with vascular tissue injury and repair." Exp Mol Pathol **66**(3): 227-237.

Metz, S., M. Wiesinger, et al. (2007). "Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP: fused receptor domains act as high affinity cytokine-binding proteins." J Biol Chem **282**(2): 1238-1248.

Michaeloudes, C., M. B. Sukkar, et al. (2011). "TGF-beta regulates Nox4, MnSOD and catalase expression, and IL-6 release in airway smooth muscle cells." Am J Physiol Lung Cell Mol Physiol **300**(2): L295-304.

Millward-Sadler, S. J., M. O. Wright, et al. (2004). "ATP in the mechanotransduction pathway of normal human chondrocytes." Biorheology **41**(3-4): 567-575.

Mitchell, P. G., H. A. Magna, et al. (1996). "Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage." J Clin Invest **97**(3): 761-768.

Miyazono, K., A. Olofsson, et al. (1991). "A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1." EMBO J **10**(5): 1091-1101.

Mogami, H., C. Lloyd Mills, et al. (1997). "Phospholipase C inhibitor, U73122, releases intracellular Ca2+, potentiates Ins(1,4,5)P3-mediated Ca2+ release and directly activates ion channels in mouse pancreatic acinar cells." Biochem J **324 (Pt 2)**: 645-651.

Morales, S. A., S. Mareninov, et al. (2007). "Collagen gel contraction by ARPE-19 cells is mediated by a FAK-Src dependent pathway." Exp Eye Res **85**(6): 790-798.

Moustakas, A. and C. H. Heldin (2005). "Non-Smad TGF-beta signals." J Cell Sci **118**(Pt 16): 3573-3584.

Mu, D., S. Cambier, et al. (2002). "The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1." J Cell Biol **157**(3): 493-507.

Munger, J. S., J. G. Harpel, et al. (1998). "Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1." Mol Biol Cell **9**(9): 2627-2638.

Munger, J. S., X. Huang, et al. (1999). "The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis." Cell **96**(3): 319-328.

Nagase, H. and J. F. Woessner, Jr. (1999). "Matrix metalloproteinases." J Biol Chem **274**(31): 21491-21494.

Nakagawa, T., J. H. Li, et al. (2004). "TGF-beta induces proangiogenic and antiangiogenic factors via parallel but distinct Smad pathways." Kidney Int **66**(2): 605-613.

Narazaki, M., K. Yasukawa, et al. (1993). "Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130." Blood **82**(4): 1120-1126.

Narouz-Ott, L., P. Maurer, et al. (2000). "Thrombospondin-4 binds specifically to both collagenous and non-collagenous extracellular matrix proteins via its C-terminal domains." J Biol Chem **275**(47): 37110-37117.

Naruse, K., X. Sai, et al. (1998). "Uni-axial cyclic stretch induces c-src activation and translocation in human endothelial cells via SA channel activation." FEBS Lett **441**(1): 111-115.

Naruse, K., T. Yamada, et al. (1998). "Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch." Am J Physiol **274**(5 Pt 2): H1532-1538.

Neame, P. J., J. E. Christner, et al. (1986). "The primary structure of link protein from rat chondrosarcoma proteoglycan aggregate." J Biol Chem **261**(8): 3519-3535.

Nomura, T. and N. Katunuma (2005). "Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells." J Med Invest **52**(1-2): 1-9.

North, R. A. (2002). "Molecular physiology of P2X receptors." Physiol Rev **82**(4): 1013-1067.

Norwitz, E. R., V. Snegovskikh, et al. (2007). "Progestin inhibits and thrombin stimulates the plasminogen activator/inhibitor system in term decidual stromal cells: implications for parturition." Am J Obstet Gynecol **196**(4): 382 e381-388.

Nowell, M. A., P. J. Richards, et al. (2003). "Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130." J Immunol **171**(6): 3202-3209.

Nunes, I., P. E. Gleizes, et al. (1997). "Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta." J Cell Biol **136**(5): 1151-1163.

Nunes, I., R. L. Shapiro, et al. (1995). "Characterization of latent TGF-beta activation by murine peritoneal macrophages." J Immunol **155**(3): 1450-1459.

Nuttall, R. K., C. J. Pennington, et al. (2003). "Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells." Mol Cancer Res **1**(5): 333-345.

Odekon, L. E., F. Blasi, et al. (1994). "Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta." J Cell Physiol **158**(3): 398-407.

Oldberg, A., P. Antonsson, et al. (1989). "A collagen-binding 59-kd protein (fibromodulin) is structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin)." EMBO J **8**(9): 2601-2604.

Oldberg, A., P. Antonsson, et al. (1992). "COMP (cartilage oligomeric matrix protein) is structurally related to the thrombospondins." J Biol Chem **267**(31): 22346-22350.

Olin, A. I., M. Morgelin, et al. (2001). "The proteoglycans aggrecan and Versican form networks with fibulin-2 through their lectin domain binding." J Biol Chem **276**(2): 1253-1261.

Ottani, V., D. Martini, et al. (2002). "Hierarchical structures in fibrillar collagens." Micron **33**(7-8): 587-596.

Paavola, M., P. Kannus, et al. (2002). "Achilles tendinopathy." J Bone Joint Surg Am **84-A**(11): 2062-2076.

Pankov, R. and K. M. Yamada (2002). "Fibronectin at a glance." J Cell Sci **115**(Pt 20): 3861-3863.

Parks, W. C. and R. P. Mecham (1998). Matrix metalloproteinases. San Diego, Calif. ; London, Academic Press.

Parreno, J. and D. A. Hart (2009). "Molecular and mechano-biology of collagen gel contraction mediated by human MG-63 cells: involvement of specific intracellular signaling pathways and the cytoskeleton." Biochem Cell Biol **87**(6): 895-904.

Pasternak, B., T. Schepull, et al. (2008). "Elevation of systemic matrix metalloproteinase-2 and -7 and tissue inhibitor of metalloproteinases-2 in patients with a history of Achilles tendon rupture: pilot study." Br J Sports Med.

Pearson, C. A., D. Pearson, et al. (1988). "Tenascin: cDNA cloning and induction by TGF-beta." EMBO J **7**(10): 2977-2982.

Perez-Castro, A. V. and K. G. Vogel (1999). "In situ expression of collagen and proteoglycan genes during development of fibrocartilage in bovine deep flexor tendon." J Orthop Res **17**(1): 139-148.

Pitts, J., E. Kam, et al. (1987). "Patterns of junctional communication in animal tissues." Ciba Found Symp **125**: 140-153.

Porter, S., I. M. Clark, et al. (2005). "The ADAMTS metalloproteinases." Biochem J **386**(Pt 1): 15-27.

Porter, S., S. D. Scott, et al. (2004). "Dysregulated expression of adamalysin-thrombospondin genes in human breast carcinoma." Clin Cancer Res **10**(7): 2429-2440.

Purslow, P. P., T. J. Wess, et al. (1998). "Collagen orientation and molecular spacing during creep and stress-relaxation in soft connective tissues." J Exp Biol **201**(Pt 1): 135-142.

Puxkandl, R., I. Zizak, et al. (2002). "Viscoelastic properties of collagen: synchrotron radiation investigations and structural model." Philos Trans R Soc Lond B Biol Sci **357**(1418): 191-197.

Qi, J., L. Chi, et al. (2007). "ATP reduces gel compaction in osteoblast-populated collagen gels." J Appl Physiol **102**(3): 1152-1160.

Qi, J., L. Chi, et al. (2009). "Modulation of collagen gel compaction by extracellular ATP is MAPK and NF-kappaB pathways dependent." Exp Cell Res **315**(11): 1990-2000.

Qi, J., J. M. Dmochowski, et al. (2012). "Differential Expression and Cellular Localization of Novel Isoforms of the Tendon Biomarker Tenomodulin." J Appl Physiol.

Ramirez, F. and H. C. Dietz (2007). "Fibrillin-rich microfibrils: Structural determinants of morphogenetic and homeostatic events." J Cell Physiol **213**(2): 326-330.

Ramirez, F. and L. Pereira (1999). "The fibrillins." Int J Biochem Cell Biol **31**(2): 255-259.

Ramirez, F. and D. B. Rifkin (2009). "Extracellular microfibrils: contextual platforms for TGFbeta and BMP signaling." Curr Opin Cell Biol **21**(5): 616-622.

Rathbun, J. B. and I. Macnab (1970). "The microvascular pattern of the rotator cuff." J Bone Joint Surg Br **52**(3): 540-553.

Ravanti, L., J. Heino, et al. (1999). "Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase." J Biol Chem **274**(4): 2446-2455.

Rawlings, N. D., A. J. Barrett, et al. (2012). "MEROPS: the database of proteolytic enzymes, their substrates and inhibitors." Nucleic Acids Res **40**(Database issue): D343-350.

Rees, S. G., C. R. Flannery, et al. (2000). "Catabolism of aggrecan, decorin and biglycan in tendon." Biochem J **350 Pt 1**: 181-188.

Ribeiro, S. M., M. Poczatek, et al. (1999). "The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta." J Biol Chem **274**(19): 13586-13593.

Riddle, R. C., A. F. Taylor, et al. (2006). "MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation." Am J Physiol Cell Physiol **290**(3): C776-784.

Riikonen, T., J. Westermarck, et al. (1995). "Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression." J Biol Chem **270**(22): 13548-13552.

Riley, G. (2004). Soft tissue rheumatology. Chapter 1.3: Tendon and Ligament biochemistry and pathology. 1st edition. Oxford, Oxford University Press.

Riley, G. (2005). "Chronic tendon pathology: molecular basis and therapeutic implications." Expert Rev Mol Med **7**(5): 1-25.

Riley, G. P., V. Curry, et al. (2002). "Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology." Matrix Biol **21**(2): 185-195.

Riley, G. P., R. L. Harrall, et al. (1996). "Tenascin-C and human tendon degeneration." Am J Pathol **149**(3): 933-943.

Riley, G. P., R. L. Harrall, et al. (1994). "Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis." Ann Rheum Dis **53**(6): 367-376.

Riley, G. P., R. L. Harrall, et al. (1994). "Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis." Ann Rheum Dis **53**(6): 359-366.

Riser, B. L., P. Cortes, et al. (1996). "Cyclic stretching force selectively up-regulates transforming growth factor-beta isoforms in cultured rat mesangial cells." Am J Pathol **148**(6): 1915-1923.

Riser, B. L., S. Ladson-Wofford, et al. (1999). "TGF-beta receptor expression and binding in rat mesangial cells: modulation by glucose and cyclic mechanical strain." Kidney Int **56**(2): 428-439.

Ritty, T. M., K. Ditsios, et al. (2002). "Distribution of the elastic fiber and associated proteins in flexor tendon reflects function." Anat Rec **268**(4): 430-440.

Robbins, J. R., S. P. Evanko, et al. (1997). "Mechanical loading and TGF-beta regulate proteoglycan synthesis in tendon." Arch Biochem Biophys **342**(2): 203-211.

Rolli, M., E. Fransvea, et al. (2003). "Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells." Proc Natl Acad Sci U S A **100**(16): 9482-9487.

Rui, Y. F., P. P. Lui, et al. (2011). "Expression of chondro-osteogenic BMPs in clinical samples of patellar tendinopathy." Knee Surg Sports Traumatol Arthrosc.

Rusch, A., C. J. Kros, et al. (1994). "Block by amiloride and its derivatives of mechano-electrical transduction in outer hair cells of mouse cochlear cultures." J Physiol **474**(1): 75-86.

Saez, J. C., V. M. Berthoud, et al. (2003). "Plasma membrane channels formed by connexins: their regulation and functions." Physiol Rev **83**(4): 1359-1400.

Saharinen, J., J. Taipale, et al. (1996). "Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1." EMBO J **15**(2): 245-253.

Salingcarnboriboon, R., H. Yoshitake, et al. (2003). "Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property." Exp Cell Res **287**(2): 289-300.

Salter, D. M., W. H. Wallace, et al. (2000). "Human bone cell hyperpolarization response to cyclical mechanical strain is mediated by an interleukin-1beta autocrine/paracrine loop." J Bone Miner Res **15**(9): 1746-1755.

Samiric, T., M. Z. Ilic, et al. (2004). "Characterisation of proteoglycans and their catabolic products in tendon and explant cultures of tendon." Matrix Biol **23**(2): 127-140.

Sanford, L. P., I. Ormsby, et al. (1997). "TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes." Development **124**(13): 2659-2670.

Sarasa-Renedo, A. and M. Chiquet (2005). "Mechanical signals regulating extracellular matrix gene expression in fibroblasts." Scand J Med Sci Sports **15**(4): 223-230.

Sasaki, T., W. Gohring, et al. (1999). "Tropoelastin binding to fibulins, nidogen-2 and other extracellular matrix proteins." FEBS Lett **460**(2): 280-284.

Sasamoto, A., M. Nagino, et al. (2005). "Mechanotransduction by integrin is essential for IL-6 secretion from endothelial cells in response to uniaxial continuous stretch." Am J Physiol Cell Physiol **288**(5): C1012-1022.

Sato, Y. and D. B. Rifkin (1989). "Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture." J Cell Biol **109**(1): 309-315.

Schindler, R., J. Mancilla, et al. (1990). "Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF." Blood **75**(1): 40-47.

Schmidt-Rohlfing, B., J. Graf, et al. (1992). "The blood supply of the Achilles tendon." Int Orthop **16**(1): 29-31.

Schultz-Cherry, S. and J. E. Murphy-Ullrich (1993). "Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism." J Cell Biol **122**(4): 923-932.

Schweitzer, R., J. H. Chyung, et al. (2001). "Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments." Development **128**(19): 3855-3866.

Sciore, P., R. Boykiw, et al. (1998). "Semiquantitative reverse transcription-polymerase chain reaction analysis of mRNA for growth factors and growth factor receptors from normal and healing rabbit medial collateral ligament tissue." J Orthop Res **16**(4): 429-437.

Scott, A., J. L. Cook, et al. (2007). "Tenocyte responses to mechanical loading in vivo: a role for local insulin-like growth factor 1 signaling in early tendinosis in rats." Arthritis Rheum **56**(3): 871-881.

Scott, A., P. Danielson, et al. (2011). "Mechanical force modulates scleraxis expression in bioartificial tendons." J Musculoskelet Neuronal Interact **11**(2): 124-132.

Scott, A., A. Sampaio, et al. (2010). "Scleraxis expression is coordinately regulated in a murine model of patellar tendon injury." J Orthop Res **29**(2): 289-296.

Screen, H. R., V. H. Chhaya, et al. (2006). "The influence of swelling and matrix degradation on the microstructural integrity of tendon." Acta Biomater **2**(5): 505-513.

Screen, H. R., D. A. Lee, et al. (2004). "An investigation into the effects of the hierarchical structure of tendon fascicles on micromechanical properties." Proc Inst Mech Eng [H] **218**(2): 109-119.

Screen, H. R., J. C. Shelton, et al. (2005). "Cyclic tensile strain upregulates collagen synthesis in isolated tendon fascicles." Biochem Biophys Res Commun **336**(2): 424-429.

Sell, D. R. and V. M. Monnier (1989). "Isolation, purification and partial characterization of novel fluorophores from aging human insoluble collagen-rich tissue." Connect Tissue Res **19**(1): 77-92.

Senga, K., M. Kobayashi, et al. (1995). "Type VI collagen in mouse masseter tendon, from osseous attachment to myotendinous junction." Anat Rec **243**(3): 294-302.

Shapiro, A. L., E. Vinuela, et al. (1967). "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels." Biochem Biophys Res Commun **28**(5): 815-820.

Sheppard, D. (2005). "Integrin-mediated activation of latent transforming growth factor beta." Cancer Metastasis Rev **24**(3): 395-402.

Shi, Y. and J. Massague (2003). "Mechanisms of TGF-beta signaling from cell membrane to the nucleus." Cell **113**(6): 685-700.

Shiomi, T., V. Lemaitre, et al. (2010). "Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases." Pathol Int **60**(7): 477-496.

Shukunami, C., A. Takimoto, et al. (2006). "Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes." Dev Biol **298**(1): 234-247.

Sironi, M., F. Breviario, et al. (1989). "IL-1 stimulates IL-6 production in endothelial cells." J Immunol **142**(2): 549-553.

Skutek, M., M. van Griensven, et al. (2001). "Cyclic mechanical stretching modulates secretion pattern of growth factors in human tendon fibroblasts." Eur J Appl Physiol **86**(1): 48-52.

Skutek, M., M. van Griensven, et al. (2003). "Cyclic mechanical stretching of human patellar tendon fibroblasts: activation of JNK and modulation of apoptosis." Knee Surg Sports Traumatol Arthrosc **11**(2): 122-129.

Smith, M. M., G. Sakurai, et al. (2008). "Modulation of aggrecan and ADAMTS expression in ovine tendinopathy induced by altered strain." Arthritis Rheum **58**(4): 1055-1066.

Smith, R. K., L. Zunino, et al. (1997). "The distribution of cartilage oligomeric matrix protein (COMP) in tendon and its variation with tendon site, age and load." Matrix Biol **16**(5): 255-271.

Sodersten, F., S. Ekman, et al. (2006). "Thrombospondin-4 and cartilage oligomeric matrix protein form heterooligomers in equine tendon." Connect Tissue Res **47**(2): 85-91.

Somerville, R. P., J. M. Longpre, et al. (2004). "ADAMTS7B, the full-length product of the ADAMTS7 gene, is a chondroitin sulfate proteoglycan containing a mucin domain." J Biol Chem **279**(34): 35159-35175.

Sood, A., D. Bynum, et al. (1999). "Gap Junction blockade inhibits calcium signalling in vitro and mechanical load induced mitogenesis and collagen cynthesis in avian tendons ex vivo " In: Transactions of the Orthopaedic research society; Anaheim, California, Orthopaedic Research Society, 1999

Stout, C. E., J. L. Costantin, et al. (2002). "Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels." J Biol Chem **277**(12): 10482-10488.

Su, S., J. A. DiBattista, et al. (1998). "Up-regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-beta in articular chondrocytes is mediated by serine/threonine and tyrosine kinases." J Cell Biochem **70**(4): 517-527.

Suh, B. C., T. D. Kim, et al. (2000). "Differential regulation of P2Y(11) receptor-mediated signalling to phospholipase C and adenylyl cyclase by protein kinase C in HL-60 promyelocytes." Br J Pharmacol **131**(3): 489-497.

Sun, H. B., N. Andarawis-Puri, et al. (2010). "Cycle-dependent matrix remodeling gene expression response in fatigue-loaded rat patellar tendons." J Orthop Res **28**(10): 1380-1386.

Sun, H. B., Y. Li, et al. (2008). "Coordinate regulation of IL-1beta and MMP-13 in rat tendons following subrupture fatigue damage." Clin Orthop Relat Res **466**(7): 1555-1561.

Sun, X., D. F. Mosher, et al. (1989). "Heparan sulfate-mediated binding of epithelial cell surface proteoglycan to thrombospondin." J Biol Chem **264**(5): 2885-2889.

Surridge, A. K., U. R. Rodgers, et al. (2009). "Characterization and regulation of ADAMTS-16." Matrix Biol **28**(7): 416-424.

Svensson, L., A. Aszodi, et al. (1999). "Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon." J Biol Chem **274**(14): 9636-9647.

Szczodry, M., J. Zhang, et al. (2009). "Treadmill running exercise results in the presence of numerous myofibroblasts in mouse patellar tendons." J Orthop Res **27**(10): 1373-1378.

Tacheau, C., J. Fontaine, et al. (2008). "TGF-beta induces connexin43 gene expression in normal murine mammary gland epithelial cells via activation of p38 and PI3K/AKT signaling pathways." J Cell Physiol **217**(3): 759-768.

Taipale, J., K. Koli, et al. (1992). "Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin." J Biol Chem **267**(35): 25378-25384.

Taipale, J., K. Miyazono, et al. (1994). "Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein." J Cell Biol **124**(1-2): 171-181.

Taipale, J., J. Saharinen, et al. (1996). "Latent transforming growth factor-beta 1 and its binding protein are components of extracellular matrix microfibrils." J Histochem Cytochem **44**(8): 875-889.

Taylor, S. E., A. Vaughan-Thomas, et al. (2009). "Gene expression markers of tendon fibroblasts in normal and diseased tissue compared to monolayer and three dimensional culture systems." BMC Musculoskelet Disord **10**: 27.

Thieszen, S. L. and T. H. Rosenquist (1995). "Expression of collagens and decorin during aortic arch artery development: implications for matrix pattern formation." Matrix Biol **14**(7): 573-582.

Tierney, G. M., N. R. Griffin, et al. (1999). "A pilot study of the safety and effects of the matrix metalloproteinase inhibitor marimastat in gastric cancer." Eur J Cancer **35**(4): 563-568.

Timpl, R., H. Rohde, et al. (1979). "Laminin--a glycoprotein from basement membranes." J Biol Chem **254**(19): 9933-9937.

Tolsma, S. S., O. V. Volpert, et al. (1993). "Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity." J Cell Biol **122**(2): 497-511.

Towbin, H., T. Staehelin, et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." Proc Natl Acad Sci U S A **76**(9): 4350-4354.

Tran, H., M. Mattei, et al. (1997). "Human fibulin-1D: molecular cloning, expression and similarity with S1-5 protein, a new member of the fibulin gene family." Matrix Biol **15**(7): 479-493.

Tsuzaki, M., D. Bynum, et al. (2005). "Mechanical loading stimulates ecto-ATPase activity in human tendon cells." J Cell Biochem **96**(1): 117-125.

Tsuzaki, M., D. Bynum, et al. (2003). "ATP modulates load-inducible IL-1beta, COX 2, and MMP-3 gene expression in human tendon cells." J Cell Biochem **89**(3): 556-562.

Uchida, H., H. Tohyama, et al. (2005). "Stress deprivation simultaneously induces over-expression of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta in fibroblasts and mechanical deterioration of the tissue in the patellar tendon." J Biomech **38**(4): 791-798.

Uitto, J. (1979). "Biochemistry of the elastic fibers in normal connective tissues and its alterations in diseases." J Invest Dermatol **72**(1): 1-10.

Uria, J. A. and C. Lopez-Otin (2000). "Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity." Cancer Res **60**(17): 4745-4751.

Valera, S., N. Hussy, et al. (1994). "A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP." Nature **371**(6497): 516-519.

Velasco, J., J. Li, et al. (2011). "Adamts5 Deletion Blocks Murine Dermal Repair through CD44-mediated Aggrecan Accumulation and Modulation of Transforming Growth Factor {beta}1 (TGF{beta}1) Signaling." J Biol Chem **286**(29): 26016-26027.

Verderio, E., C. Gaudry, et al. (1999). "Regulation of cell surface tissue transglutaminase: effects on matrix storage of latent transforming growth factor-beta binding protein-1." J Histochem Cytochem **47**(11): 1417-1432.

Visse, R. and H. Nagase (2003). "Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry." Circ Res **92**(8): 827-839.

Vogel, K. G. and D. Heinegard (1985). "Characterization of proteoglycans from adult bovine tendon." J Biol Chem **260**(16): 9298-9306.

Vogel, K. G. and T. J. Koob (1989). "Structural specialization in tendons under compression." Int Rev Cytol **115**: 267-293.

Voloshin, I., J. Gelinas, et al. (2005). "Proinflammatory cytokines and metalloproteases are expressed in the subacromial bursa in patients with rotator cuff disease." Arthroscopy **21**(9): 1076.

Walia, B., L. Wang, et al. (2003). "TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2." FASEB J **17**(14): 2130-2132.

Wall, M. E. and A. J. Banes (2004). "Mechanically induced strain upregulates connexin 43 mRNA expression in tendon cells." In: Transactions of the Orthopaedic research society; San Francisco, Orthopaedic Research Society, 2004.

Wall, M. E. and A. J. Banes (2005). "Early responses to mechanical load in tendon: role for calcium signaling, gap junctions and intercellular communication." J Musculoskelet Neuronal Interact **5**(1): 70-84.

Wall, M. E., C. Otey, et al. (2007). "Connexin 43 is localized with actin in tenocytes." Cell Motil Cytoskeleton **64**(2): 121-130.

Wang, J. H. (2006). "Mechanobiology of tendon." J Biomech **39**(9): 1563-1582.

Wang, J. H., F. Jia, et al. (2003). "Cyclic mechanical stretching of human tendon fibroblasts increases the production of prostaglandin E2 and levels of cyclooxygenase expression: a novel in vitro model study." Connect Tissue Res **44**(3-4): 128-133.

Wang, L. H., R. A. Kirken, et al. (1999). "JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response." J Immunol **162**(7): 3897-3904.

Wang, N., J. D. Tytell, et al. (2009). "Mechanotransduction at a distance: mechanically coupling the extracellular matrix with the nucleus." Nat Rev Mol Cell Biol **10**(1): 75-82.

Wang, P., M. Tortorella, et al. (2004). "Proprotein convertase furin interacts with and cleaves pro-ADAMTS4 (Aggrecanase-1) in the trans-Golgi network." J Biol Chem **279**(15): 15434-15440.

Wang, W. M., S. Lee, et al. (2003). "Transforming growth factor-beta induces secretion of activated ADAMTS-2. A procollagen III N-proteinase." J Biol Chem **278**(21): 19549-19557.

Wang, Z., R. Juttermann, et al. (2000). "TIMP-2 is required for efficient activation of proMMP-2 in vivo." J Biol Chem **275**(34): 26411-26415.

Watanabe, H., M. P. de Caestecker, et al. (2001). "Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells." J Biol Chem **276**(17): 14466-14473.

White, L. A., T. I. Mitchell, et al. (2000). "Transforming growth factor beta inhibitory element in the rabbit matrix metalloproteinase-1 (collagenase-1) gene functions as a repressor of constitutive transcription." Biochim Biophys Acta **1490**(3): 259-268.

Wiberg, C., D. Heinegard, et al. (2002). "Biglycan organizes collagen VI into hexagonal-like networks resembling tissue structures." J Biol Chem **277**(51): 49120-49126.

Wicks, S. J., S. Lui, et al. (2000). "Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II." Mol Cell Biol **20**(21): 8103-8111.

Wight, T. N. (2002). "Versican: a versatile extracellular matrix proteoglycan in cell biology." Curr Opin Cell Biol **14**(5): 617-623.

Will, H., S. J. Atkinson, et al. (1996). "The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3." J Biol Chem **271**(29): 17119-17123.

Wipff, P. J., D. B. Rifkin, et al. (2007). "Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix." J Cell Biol **179**(6): 1311-1323.

Woessner, J. F., Jr. (1991). "Matrix metalloproteinases and their inhibitors in connective tissue remodeling." FASEB J **5**(8): 2145-2154.

Wolf, F. W., R. L. Eddy, et al. (1990). "Structure and chromosomal localization of the human thrombospondin gene." Genomics **6**(4): 685-691.

Woo, S. L. Y., P. Renström, et al. (2007). Tendinopathy in athletes. Oxford, Blackwell.

Worthington, J. J., J. E. Klementowicz, et al. (2011). "TGFbeta: a sleeping giant awoken by integrins." Trends Biochem Sci **36**(1): 47-54.

Wright, C. S., M. A. van Steensel, et al. (2009). "Connexin mimetic peptides improve cell migration rates of human epidermal keratinocytes and dermal fibroblasts in vitro." Wound Repair Regen **17**(2): 240-249.

Wurgler-Hauri, C. C., L. M. Dourte, et al. (2007). "Temporal expression of 8 growth factors in tendon-to-bone healing in a rat supraspinatus model." J Shoulder Elbow Surg **16**(5 Suppl): S198-203.

Xu, Y., S. Gurusiddappa, et al. (2000). "Multiple binding sites in collagen type I for the integrins alpha1beta1 and alpha2beta1." J Biol Chem **275**(50): 38981-38989.

Yamamoto, K., R. Korenaga, et al. (2000). "Fluid shear stress activates Ca(2+) influx into human endothelial cells via P2X4 purinoceptors." Circ Res **87**(5): 385-391.

Yamanishi, Y., D. L. Boyle, et al. (2002). "Expression and regulation of aggrecanase in arthritis: the role of TGF-beta." J Immunol **168**(3): 1405-1412.

Yang, G., R. C. Crawford, et al. (2004). "Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions." J Biomech **37**(10): 1543-1550.

Yang, G., H. J. Im, et al. (2005). "Repetitive mechanical stretching modulates IL-1beta induced COX-2, MMP-1 expression, and PGE2 production in human patellar tendon fibroblasts." Gene **363**: 166-172.

Yang, Z., Z. Mu, et al. (2007). "Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice." J Cell Biol **176**(6): 787-793.

Yegutkin, G., P. Bodin, et al. (2000). "Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5'-nucleotidase along with endogenous ATP from vascular endothelial cells." Br J Pharmacol **129**(5): 921-926.

Yehualaeshet, T., R. O'Connor, et al. (1999). "Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36." Am J Pathol **155**(3): 841-851.

You, J., C. R. Jacobs, et al. (2002). "P2Y purinoceptors are responsible for oscillatory fluid flow-induced intracellular calcium mobilization in osteoblastic cells." J Biol Chem **277**(50): 48724-48729.

Yu, Q. and I. Stamenkovic (2000). "Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis." Genes Dev **14**(2): 163-176.

Zeng, G., H. M. McCue, et al. (1996). "Endogenous TGF-beta activity is modified during cellular aging: effects on metalloproteinase and TIMP-1 expression." Exp Cell Res **228**(2): 271-276.

Zhang, J., W. Li, et al. (2003). "Regulation of the intestinal epithelial response to cyclic strain by extracellular matrix proteins." FASEB J **17**(8): 926-928.

Zhang, W. M., J. Kapyla, et al. (2003). "alpha 11beta 1 integrin recognizes the GFOGER sequence in interstitial collagens." J Biol Chem **278**(9): 7270-7277.

Zhang, X. L., N. Topley, et al. (2005). "Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling." J Biol Chem **280**(13): 12239-12245.

Zheng, X., D. Chung, et al. (2001). "Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura." J Biol Chem **276**(44): 41059-41063.