MEDIATORS OF FIBROSIS IN VITRO AND IN AN IN VIVO RAT MODEL OF REPETITIVE OVERUSE INJURY

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ABSTRACT

Key clinical features of Work-Related Musculoskeletal Disorders (WMSDs) include pain, tendinosis and tissue fibrosis, although the etiology is still under investigation. Here, we characterized expressions of key mediators of fibrosis in an *in vivo* rat model of repetitive overuse injury and the effects of these mediators on fibroblast proliferation and collagen deposition *in vitro*. We first evaluated levels of anti-fibrogenic and fibrogenic proteins in young adult female rats performing high repetitive high force (HRHF) reaching and grasping task for 18 weeks. The anti-fibrogenic protein IFNγ increased at week 18 in muscle. Transforming growth factor beta-1 (TGFß-1) and CCN2 (formerly known as connective tissue growth factor, or CTGF) also increased in and around flexor digitorum muscles and extracellular matrix in the mid-forearm at 18 weeks, suggesting that TGFß-1 may serve as a bio-target to inhibit fibrosis development occurring as a consequence of WMSDs.

TGFß-1 and CCN2 are important mediators of tissue fibrosis by their stimulatory effect on extracellular matrix deposition, with CCN2 functions as a downstream mediator of TGFß-1. Substance P (SubP), a neuropeptide, has also been linked to tissue fibrosis, although little work has been done to understand whether SubP directly causes fibrotic responses in tenocytes. In the *in vitro* study, we used primary tenocytes (obtained from flexor digitorum tendons of normal young adult female rats) and a cell line of primary rat dermal fibroblasts (RDF cells). We hypothesized that SubP either acts directly or indirectly through CCN2 or acts independently to increase fibroblast proliferation and extracellular matrix production *in vitro*. We demonstrated that SubP and TGFß-1 can

induce CCN2 production in both rat tenocytes and RDF cells. Our data indicated that SubP-induced CCN2 production may be TGFß-1 independent since no TGFß-1 expression was detected after SubP treatment. We further demonstrated that SubP and TGFß-1 did not act in an additive or synergistic fashion to promote fibroblast proliferation and collagen production. Our findings suggest that both SubP and TGFß-1 have distinct fibrotic actions on tenocytes and both may be involved in tendinosis observed in animal models and patients.

DEDICATION

To my parents:
Zhongnan Zhao and Jianyue Li

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LIST OF ABBREVIATIONS

Abbreviation	Definition
APCs	Antigen-presenting cells
BCA	Bicinchoninic acid
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
ß-actin	Beta-actin
C	Control
CCN2/CTGF	Connective tissue growth factor
COL1	Collagen type I
CT	Connective Tissue
CTS	Carpal tunnel syndrome
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
FBS	Fetal bovine serum
FD	Flexor Digitorum
FGF-2	Fibroblast growth factor-2
FRC	Food restricted control
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HRHF	High repetition high force
HRLF	High repetition low force
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1beta	Interleukin-1 beta
IL-6	Interleukin-6
IGF-1	Insulin-like growth factor-1
IR	Immunoreactive
M-CSF	macrophage colony-stimulating factor
MMPs	Matrix metalloproteases
MSD	Musculoskeletal disorder
NC	Normal control
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PNS	Peripheral nervous system

PRP	Platelet-rich plasma
RDF	Rat dermal fibroblast
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SubP	Substance P
TBS	Tris Buffered Saline
TGFß-1	Transforming growth factor beta-1
TNF-alpha	Tumor necrosis factor alpha
TRHF	Trained to high-force
UE	Upper extremity
VEGF	Vascular endothelial growth factor
WB	Western Blot
WMSD	Work-related musculoskeletal disorders
CCN2/CTGF	Connective tissue growth factor
IFNγ	Interferon gamma
MIP	Macrophage inflammatory protein
TR rats	Trained rats
α-SMA	Alpha-smooth muscle actin

CHAPTER 1

INTRODUCTION

Work-Related Musculoskeletal Disorders (WMSDs)

According to the United States Occupational Safety and Health Administration (OSHA), WMSDs account for over 600,000 injuries and illnesses (OSHA, 2014), and 34 percent of all lost workdays (BLS, 2013). These disorders are estimated at $20 billion in direct costs yearly. Work-related musculoskeletal disorders (WMSDs) are also known as ergonomic injuries, repetitive strain injuries, and overuse injuries. These disorders are injuries that frequently cause disability and impairment of the muscles, nerves, tendons, joints, cartilages and spinal discs associated with exposure to risk factors in the workplace (Piligian et al., 2000). WMSDs include sprains, strains, tears, edema, fractures, compression, malalignment, disk herniation or excessive repetitive movements that challenge the musculoskeletal, connective or neural tissue reactions (e.g. carpal tunnel syndrome) occurring as the result of stressful lifting, bending, climbing, crawling, reaching, twisting, pushing, pulling, poor postural alignment, psychological stress, overexertion, or repetition (Gallagher and Heberger, 2013).

The risk factors associated with the development or exacerbations of WMSDs in the workplace include one or more conditions of physical, biomechanical, environmental or specific job demands and/or psychosocial stress in the work place, such as repetitive motion, awkward posture and vibration (OSHA, 2014). In addition, certain worker characteristics may predispose individuals to WMSDs, including obesity, poor nutrition/hydration, metabolic disease, personality and/or coping mechanisms. WMSDs

are now considered a leading cause of long-term pain and physical disability worldwide (OSHA, 2014), with diagnoses including tendinopathies, nerve compression syndromes, and muscular and joint disorders. Studies in human with upper extremity WMSDs find

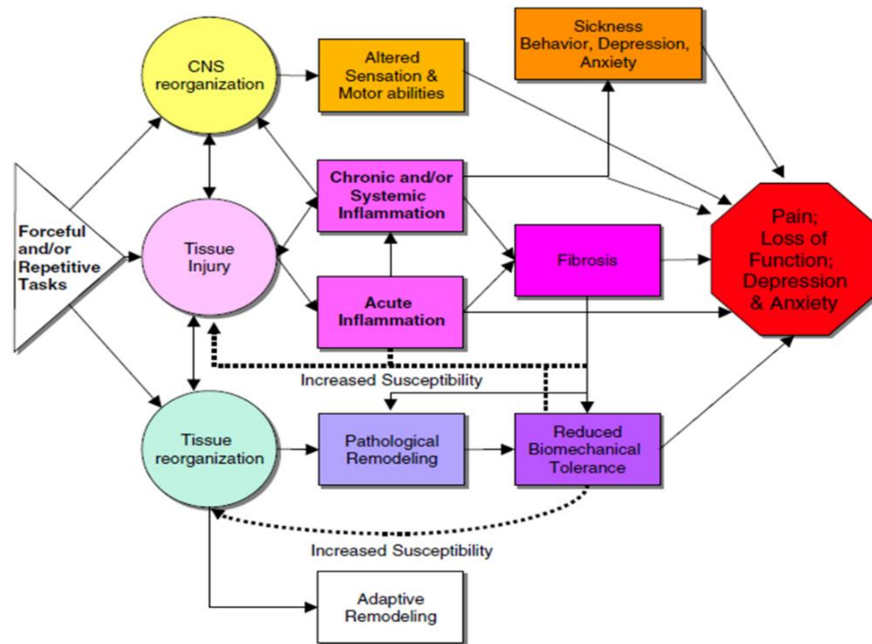

Figure 1-1. Diagram showing three primary pathways hypothesized to lead to work-related musculoskeletal disorders caused by repetitive and/or forceful hand-intensive tasks. CNS reorganization, tissue injury, or tissue reorganization. With respect to the injury/inflammatory pathway, these authors hypothesized that repetitive tasks lead to tissue injury, then acute inflammation. Normally, this response would activate cellular mechanisms related to wound healing. However, with overuse MSDs, the continued cycle of tissue trauma induced by continued performance of a repetitive and/ or forceful task hinders repair. Instead, a chronic inflammatory response is stimulated along with or inducing a fibrogenic response. Continued performance of high demand tasks can also lead to structural disorganization (reduced biomechanical tolerance and pathological remodeling). However, if task exposure is low enough to avoid tissue injury, then inflammation is avoided and tissue reorganization moves into a beneficial adaptive remodeling. What is potentially intriguing about this graded response of musculoskeletal and peripheral nerve tissues to repetitive motion is the possibility that there is a threshold of activity below which the tissue response, whether accompanied by inflammation or not, leads to adaptive rather than degenerative long-term tissue changes. Possible interrelationships between components of these pathways are indicated, which illustrates the pathomechanical complexity that may contribute to an end point of pain, loss of function. *(From Barbe et al. Brain Behav Immun. 2006 Sep;20(5):423-9. Figure used with permission from Copyright Clearance Center).*

evidence of inflammation, fibrosis and/or degeneration in serum, nerves and musculotendinous tissues, depending on the stage of disorder in a particular individual (Barbe and Barr, 2006; Carp et al., 2007; Rechardt et al., 2011). However, the mechanistic link between these processes is unclear, although some authors have postulated possible links and interactions (Fig. 1-1) (Barbe and Barr, 2006).

Wound Healing Overview

Wound healing is the process by which damaged tissue attempts to restore normal tissue architecture and function after an injury in order to restore homeostasis. There are a series of molecular, vascular and cellular responses involved in this process (Diegelmann and Evans, 2004). Wound healing is not linear and can be divided into three distinct phases: 1) hemostasis/acute inflammation, 2) proliferation/fibroplasia, and 3) remodeling/maturation (Fig. 1-2).

Unfortunately, many factors can disrupt wound healing. For example, if the injury or stimulus is repeated or chronic, there is a greater possibility that tissues can develop chronic inflammation and fibrosis. In these cases, chronic or cyclic release of a variety of inflammatory or fibrogenic biochemical mediators can perpetuate the inflammatory response or cause excessive proliferation and fibroplasia.

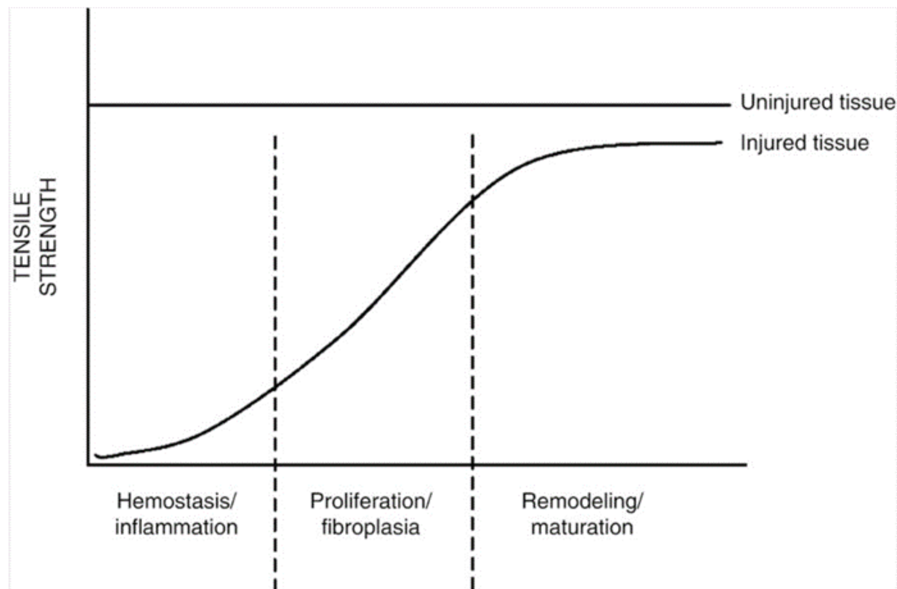

Figure 1-2. Wound healing response in tendons after an acute injury. The hemostasis/acute inflammation phase is the natural response of the body to injury. During this phase, macrophages and neutrophils play an important role in phagocytosis by cleaning up dead cells and tissue debris. In the proliferation/fibroplasia phase, which normally lasts 2 days to 3 weeks, fibroblasts proliferate and lay a bed of collagen. New blood vessels and the granulation tissue accumulate which is comprised of collagen and extracellular matrix at the wound area. The remodeling/maturation phase is the final phase and involves remodeling of the newly deposited collagen from type III to type I. Simultaneously, cellular activity reduces and the number of blood vessels in the wound area decreases. *(From Lin TW, Cardenas L, Soslowsky LJ: Biomechanics of tendon injury and repair, J Biomech 37:866, 2004. Figure used with permission from Copyright Clearance Center).*

Tissue Injury

Tissue injury can result from various stimuli, including infections, mechanical injuries and autoimmune reactions. Wound healing, the intricate process where the tissue repairs itself after injury, is a fundamental process that allows the replacement of damaged or injured cells after infection, mechanical loading or injuries (Fig.1-2 shows the mechanism of tendon repair after an acute injury).

Wound Healing: Regenerative Versus Fibrosis Repair

Although initially beneficial, the wound healing process can become pathogenic if it continues unchecked, which would result in chronic long-term inflammatory responses, followed by sustained fibroblast proliferation and extracellular matrix production. One of the biggest outcomes of chronic WMSDs is the development of tissue fibrosis, which is often defined as the wound healing process that has gone out of control (Abdelmagid et al., 2012; Barbe et al., 2013a).

When injuries occur, a hemostasis/acute inflammation phase is induced (Fig. 1-2). During this phase, epithelial or endothelial cells release inflammatory cytokines that initiate an anti-fibrinolytic-coagulation cascade, which triggers extravascular coagulation (Fig. 1-3) (Wynn, 2007). This is followed by innate immune activation, fibroblast recruitment, proliferation and activation; and extracellular matrix (ECM) synthesis and cross-linking (the proliferation/fibroplasia phase shown in Fig. 1-2). In detail, leukocytes are recruited and then activated and proliferate in response to chemokines and growth factors produced by epithelial and endothelial cells. Stimulated epithelial cells, endothelial cells and fibroblasts then produce matrix metalloproteinases (MMPs), which disrupt basement membranes, allowing the recruitment of inflammatory cells to the site of injury. During the initial phase of leukocytes migration, activated macrophages and neutrophils clean up tissue debris, dead cells and any invading organisms. The activated leukocytes secrete pro-fibrotic cytokines such as interleukin 13 (IL-13) and transforming growth factor beta 1 (TGFß-1) (Wynn, 2003; Li et al., 2006), which in turn activate macrophages and fibroblasts. Activated fibroblasts transform into alpha-smooth muscle

actin (α-SMA) - expressing myofibroblasts. Shortly after the initial inflammatory phase, myofibroblasts produce ECM components and endothelial cells form new vessels.

Thereafter, there are two primary outcomes associated with wound healing: 1) complete resolution with total restoration of normal tissue structure and strength (depicted as the remodeling/maturation phase in Fig. 1-2); or 2) repair with scar formation of varying degrees, depending on the level of injury. In the case of regeneration, collagen fibers are more organized, blood vessels are reformed and scar tissue is eliminated, leaving no lasting evidence of damage (Lin et al., 2004). However, with repeated injuries, which last for several weeks or months, the normal wound healing process is disrupted, and persistent inflammation, tissue necrosis or fibroblast proliferation commence (Fig. 1-1) (Barbe and Barr, 2006). Some cytokines secreted by injured cells and macrophages are fibrogenic, such as CCN2 (formerly known as connective tissue growth factor, or CTGF) and TGFß-1 (Barbe et al., 2013b). Over-expression of fibrogenic mediators can lead to excessive fibroblast proliferation and matrix deposition at the wound site, a process called *fibrosis*. In some cases, tissue fibrosis may lead to organ failure and even death. In the case of tendons, a fibrotic scar often fills the tissue gap. As a consequence, an injured tendon may never return to normal structural or biomechanical properties, even after long periods of recovery (Derby et al., 2012; Thomopoulos et al., 2015).

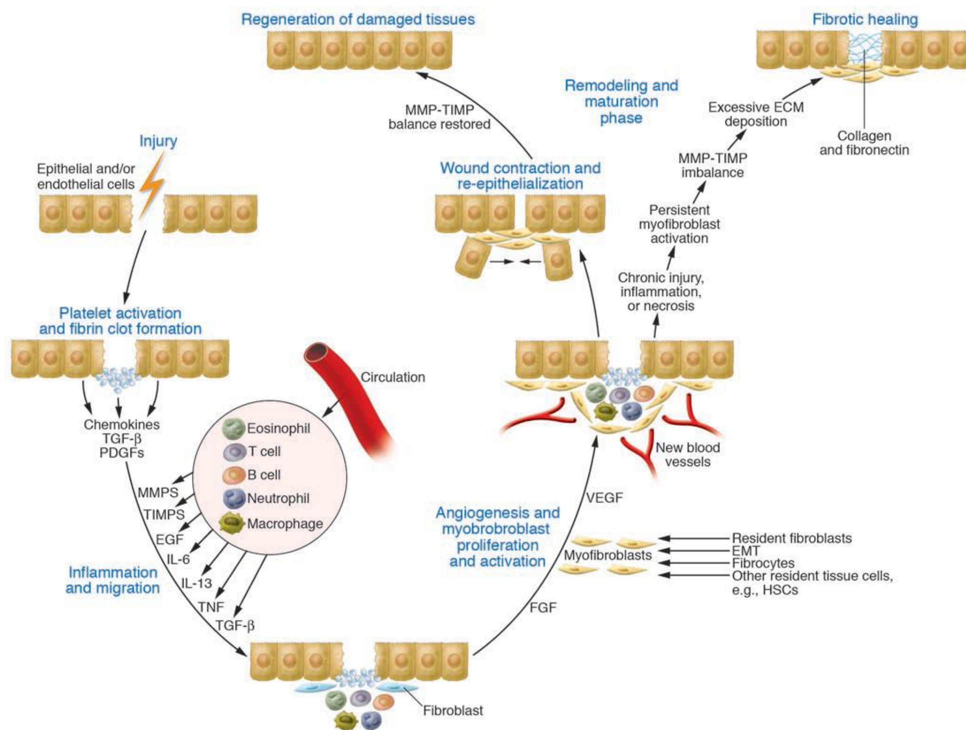

Figure 1-3. Outcomes of wound healing: tissue regeneration versus fibrosis. Following tissue injury, epithelial and/or endothelial cells release inflammatory mediators that initiate an antifibrinolytic-coagulation cascade, which triggers blood clot formation. This is followed by an inflammatory and proliferative phase, when leukocytes are recruited and then activated and induced to proliferate by chemokines and growth factors. The activated leukocytes secrete profibrotic cytokines such as TGFß-1. Stimulated epithelial cells, endothelial cells, and myofibroblasts also produce MMPs, which disrupt the basement membrane, and additional cytokines and chemokines that recruit and activate neutrophils, macrophages, T cells, B cells, and eosinophils, important components of reparative tissue. The activated macrophages and neutrophils clean up tissue debris, dead cells, and invading organisms. Shortly after the initial inflammatory phase, myofibroblasts produce ECM components, and endothelial cells form new blood vessels. In the subsequent remodeling and maturation phase, the activated myofibroblasts stimulate wound contraction. Collagen fibers also become more organized, blood vessels are restored to normal, scar tissue is eliminated, and epithelial and/or endothelial cells divide and migrate over the basal layers to regenerate the epithelium or endothelium, respectively, restoring the damaged tissue to its normal appearance. However, in the case of chronic wounds, the normal healing process is disrupted. Persistent inflammation, tissue necrosis, and infection lead to chronic myofibroblast activation and excessive accumulation of ECM components, which promotes the formation of a permanent fibrotic scar. *(From Wynn TA. 2007. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. The Journal of clinical investigation 117:524-529. Figure used with permisson from Copyright Clearance Center).*

Characteristic Features of Muscle Tissue

There are three types of muscle tissue: skeletal, cardiac, and visceral (smooth) muscle. Skeletal muscle is found attached to the bones for movement. In skeletal muscle, each muscle cell, more commonly called a muscle fiber, or myofiber, is actually a multi-nucleated syncytium. They acquired this characteristic because they develop from the fusion of small single cells into long units. Skeletal muscle cells are independent cells separated from one another by connective tissue. The connective tissue that surrounds both individual muscle fibers and bundles of muscle fibers is essential for force transduction. At the end of muscle, the connective tissue continues as a tendon or other arrangement of collagen fibers that attaches the muscle, usually to bone. A rich supply of blood vessels and nerves travels in the connective tissue (Ross MH, 1989). The connective tissue associated with muscle is named accordingly to its relationship with the muscle fibers (Fig. 1-4).

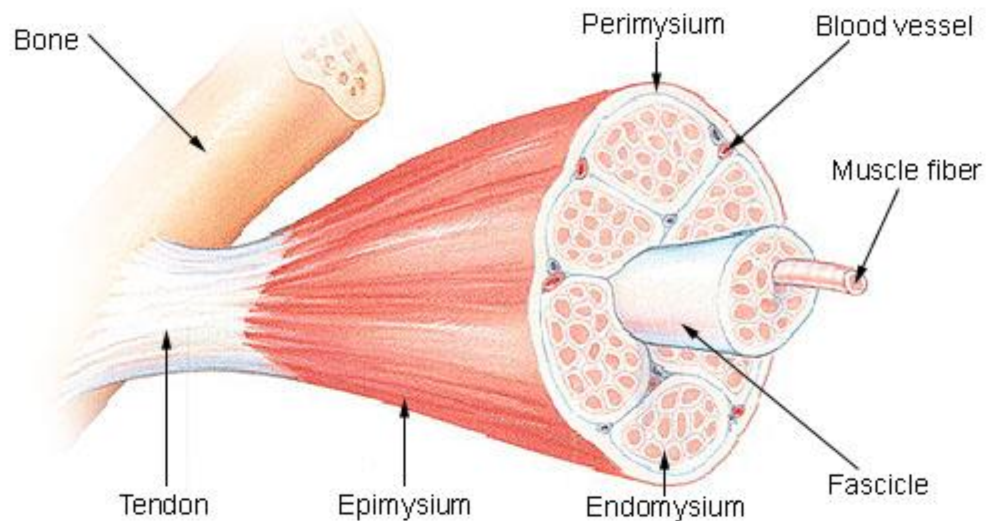

Figure 1-4. General organization of skeletal muscle. The endomysium surrounds individual muscle cells (fibers), the perimysium surrounds a muscle fascicle, and the epimysium surrounds the entire muscle. At the end of muscle, the connective tissue continues as a tendon that usually attaches to bone.
(http://en.wikipedia.org/wiki/File:Illu_muscle_structure.jpg; in the public domain)

As shown in Fig. 1-4, endomysium is the delicate layer of reticular fibers that immediately surrounds individual fibers. Perimysium is a thicker connective tissue layer that surrounds a group of fibers to form a bundle or fascicle. Epimysium is the sheath of dense connective tissue that surrounds a collection of fascicles that constitutes the muscle (Paparo, 1988).

Although not part of this study, to complete this section, cardiac muscle is a type of muscle found in the walls of the heart and in the base of the large veins that empty into the heart. Visceral muscle (smooth muscle) is found in the body's internal organs and blood vessels. It is usually called smooth muscle because it has no striations and is therefore smooth in appearance (Paparo, 1988).

Characteristic Features of Tendon Tissue

Tendon tissue is the essential connection between bone and muscle (Aslan et al., 2008) and is categorized as dense connective tissue. Every tendon has a structural hierarchy. The very outside is tertiary bundles, followed by secondary bundles (fascicles), fibrils, primary bundles (subfasicles), collagen fibers and collagen fibrils (Fig. 1-5). The epitenon and the paratenon (together called peritenon) surround the whole tendon while the endotenon runs through the tertiary fiber bundle and the fascicle (Fig. 1-5).

At the molecular level, tendon can be divided into a cellular part and an extracellular matrix (ECM) part. Tenocytes and their progenitors, tenoblasts, account for 90-95% of the cellular part (Chuen et al., 2004), and produce the ECM contents. They have been identified in the tendon sheath, paratenon, epitenon and endotenon. Normally, tenocytes stay in quiescent stage. Under pathophysiological conditions, tenocytes become activated and synthesize matrix proteins with a high metabolic activity, such as collagen type I (Riley, 2004). In addition to tenocytes, tendons contain the synovial cells of tendon sheaths, chondrocytes, vascular cells in capillaries, smooth muscle cells, adipocytes and nerve cells (Pauly et al., 2010; Backman et al., 2011b). Collagen type I is the most abundant molecule in the ECM (Riley, 2004), accounting for almost 60% of the dry mass of the tissue and approximately 95% of the total collagen. Collagen fibrils are the basic units of tendon. Other components include collagen type III, IV, V, VI, elastin and proteoglycans (Riley, 2004).

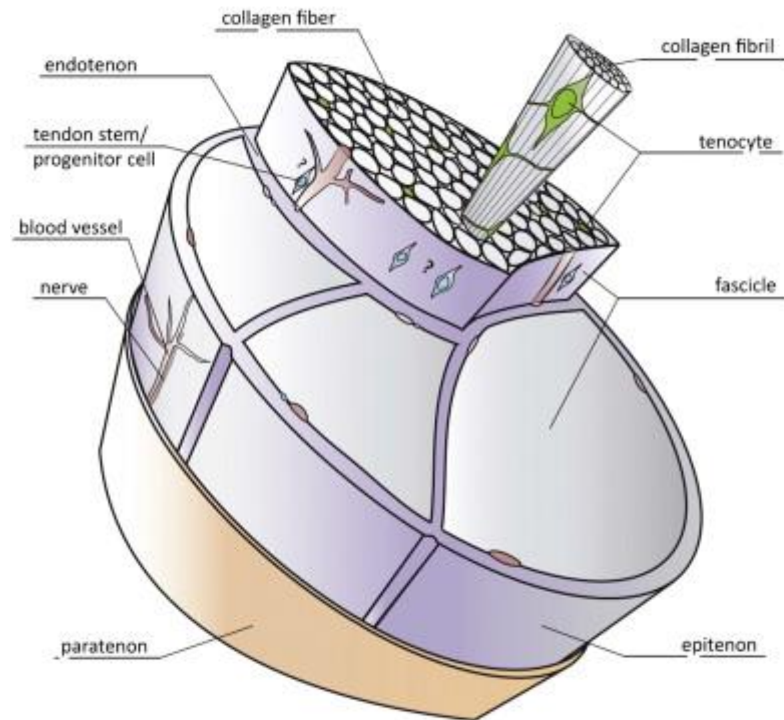

Figure 1-5. Structural hierarchy of tendons. The collagen molecules are organized hierarchically in fibrils, fibers and fascicles. The collagen fibril is the smallest unit of tendon that can be mechanically tested. Collagen fibrils form collagen fibers. The cellular components are mostly tenocytes, which are terminally differentiated cells. Different sheets, endotenon, epitenon and paratenon are shown as well as blood vessels and nerves. *(From Biologics for tendon repair. Advanced Drug Delivery Reviews. Figure used with permission from Copyright Clearance Center).*

Fibroblasts and in vitro Culturing of Tenocytes

Fibroblasts are the cells that make up the structural framework or stroma composed of the ECM and collagen in animal tissues (Ross MH, 1989). These cells are the most common type of connective tissue in animals, and are important for wound healing. Similarities and differences exist between fibroblasts of different tissue origin. For example, dermal fibroblasts are derived from mesenchymal stem cells, are cells within the dermis layer of skin, and are responsible for generating connective tissue and allowing the skin to recover from injury (Sorrell and Caplan, 2004). However, they do

not appear to be fully differentiated or specialized (Shamis et al., 2011). They can further give rise to myofibroblasts, which display high levels of smooth muscle actin and play a critical role in wound healing and fibrosis (Alberts B, 2002). In contrast, the constitutive cells of tendons, tendon fibroblasts, are more typically called tenocytes and are highly specialized elongated fibroblasts present in tendons. They are located between the collagenous tendon fibers and are responsible for synthesizing the ECM, inducing assembly of early collagen fibers, which are the basic units of the tendon (Ross MH, 1989).

Concerning *in vitro* culturing of tenocytes, a critical aspect is to isolate them from tendon tissue and preserve them in a differentiated cell state. There are several different methods used to culture tenocytes, methods that are summarized in Table 1-1. Types of enzymatic digestion and serum conditions vary from protocol to protocol, yet are critical for primary culture of tenocytes. For example, Seluanov et al digested skin fibroblasts with 0.14 Wunsch units/mL Liberase Blendzyme 3 and cultured them in 15% fetal bovine serum (FBS) contained media (Seluanov et al., 2010). In contrast, Poppe et al, Klatte et al and Backman et al used collagenase (1.5 mg/ml, 0.3% and 2mg/ml respectively) to isolate human tenocytes before culturing them in 10% FBS (Pauly et al., 2010; Poppe, 2010; Backman et al., 2011b). Luo et al collected rat tenocytes and mechanically disrupted the tissue by cutting the tendons into pieces of approximately 1 mm^3 in size (Luo et al., 2009) (Table 1-1). In the project described in Chapter 3, we utilized methods similar to Backman et al 2011.

Table 1-1. Summary of the methodology of tenocytes isolation

Author	Year	Cell type	Enzyme	Digestion (37 ° C)	Centrifuge	FBS%	Transfer tissues to new plates	After 14 days from the beginning of isolation
Seluanov	2010	Skin fibroblasts from rodents	0.14 Wunsch units/mL Liberase Blendzyme 3	Stir slowly; 30-90 minutes at 37℃	524 x g 5 min	15%	YES; if not many fibroblasts attached	Plate cells at 50,000 cells/ plate EMEM with FBS+A/A
Poppe (Dissertation)	2010	Human tenocytes	1.5 mg/ml Collagenase II	16-20 h (depending on the sizes), filter though a 70um mesh	1000 rpm 5 min	10%	Pellet only	Cells were counted and plated in flasks at 30-40% density
Klatte	2010	Human tenocytes	0.3% Collagenase type CLS II in PBS with Ca/Mg	2 hours at 37℃	not given	10%	No	When nearly confluent, cells may be passed or frozen
Luo	2009	Rat tenocytes from Achille tendons	The 50 ml flask with 1 mm tissue pieces was left upside down in incubator with 3 ml media and turned around after 24 h.			10%	No	Colonies : within 10 days; passage: after 15 days.
Backman	2011	Human tenocytes	Collagenase at 2mg/mL (Clostridopeptidase A, C-0130 Sigma)	not given	800 x g 5 min	10%	Pellet only	grown to confluence, changing media every 3-4 days

Key Factors Involved in Tissue Fibrosis

Transforming Growth Factor beta 1

TGFß-1 is a ubiquitously expressed cytokine belonging to a large superfamily of bone morphologic proteins (Shi and Massagué, 2003). TGFß-1 plays an active role in various bioactivities including proliferation and wound healing (O'Kane and Ferguson, 1997) and synthesis of ECM molecules (Hocevar and Howe, 2000). Although TGFß-1 plays an important role in tissue repair, overexpression of this protein can lead to excessive, uncontrolled depositing fibrous tissue. TGFß-1 is known to strongly contribute to fibrotic disorders, such as diabetic nephropathy, rheumatoid arthritis, myocarditis and tendinopathies (Fu et al., 2002). Increased TGFß-1 expression has been observed in patients with pulmonary, kidney and liver fibrosis (Blobe et al., 2000). TGFß-1 stimulates the proliferation and/or synthesis of ECM components in cultures of fibroblasts obtained from skin and lung (Fine and Goldstein, 1987) and stimulates collagen synthesis in pancreatic and liver fibroblasts (Ignotz and Massagué, 1986). This growth factor also regulates various processes in tendon healing, and has been suggested to play a vital role in switching the healing process from a normal one into a pathological one (Blobe et al., 2000). It is also known to modulate the inflammatory responses in early healing stages, participate in angiogenesis, regulate the proteoglycan deposition, and stimulate the production of ECM components by tenocytes (Fu et al., 2005). Increased TGFß-1 levels are associated with tendinosis, defined as a chronic injury of failed healing in tendons (Fu et al., 2002). With regard to WMSDs, tissues collected from humans and animal models with these disorders show increased TGFß-1 in conjugation with tendon and muscle fibrosis (Schild and Trueb, 2002; Smith et al., 2007; Rechardt et al., 2011; Abdelmagid et

al., 2012; Gao et al., 2013; Chikenji et al., 2014). Thus, TGFß-1 has become a target for several potential anti-fibrotic therapies.

Connective Tissue Growth Factor

Connective tissue growth factor is widely known as CTGF or CCN2. This cytokine is a 38kDa, cysteine-rich, matricellular protein. It belongs to the CCN protein family (Cyr61, CCN2, Nov, WISP1, WISP2 and WISP3) (Leask and Abraham, 2006). CCN2 is a secreted protein with major roles in angiogenesis, chondrogenesis, osteogenesis, tissue repair, cancer and fibrosis (Perbal, 2001; Perbal, 2004; De Winter et al., 2008; Cicha and Goppelt-Struebe, 2009). CCN2 is also involved in promoting complex bioactivities including proliferation, migration, adhesion, survival, differentiation and the biosynthesis of ECM proteins depending on the involved cell types (Perbal, 2004; Leask and Abraham, 2006; Chen and Lau, 2009; Cicha and Goppelt-Struebe, 2009). CCN2 is a downstream mediator of TGFß-1 induced cell proliferation, migration, adhesion and extracellular matrix production in diverse cell types, predominantly in fibrotic disorders (Grotendorst, 1997; Wang et al., 2011). CCN2 has been linked to the pathogenesis of tissue fibrosis when increased. CCN2 induces fibroblast proliferation and ECM production. Studies have been shown that CCN2 is upregulated in different human kidney diseases and contributes to renal fibrosis (Phanish et al., 2010). Studies in experimental liver fibrosis found inhibition of CCN2 expression by antisense mRNA or by CCN2-binding antibodies prevents increased collagen synthesis in fibroblasts exposed to TGFß-1, indicating that CCN2 is essential for the fibrotic response to TGFß-1 (Grotendorst, 1997; Leask and Abraham, 2004). Tenocytes and fibroblasts in tendon tissues collected from muscles and tendon tissues from animal

models of WMSDs under chronic overload conditions or injuries showed increased expression of CCN2 (Abdelmagid et al., 2012; Gao et al., 2013). In addition, TGFß-1 and CCN2 are overexpressed in a synergic fashion in many animal models of wound repair, suggesting that TGFß-1-induced CCN2 expression is involved in wound healing (Igarashi et al., 1993; Abdelmagid et al., 2012; Gao et al., 2013; Abrahams et al., 2014). Since CCN2 is normally low and undetectable in serum of healthy individuals (Barbe et al., 2013b; Gao et al., 2013), it may serve as predictive biomarker for patients in the fibrotic stage of WMSDs.

Interferon Gamma

This factor, which we will abbreviate as IFNγ, is a type II IFN family cytokine. It was originally discovered as an anti-viral agent and is known to be produced by many immune cell types such as CD4+ Th1 and CD8+ cytotoxic T lymphocytes, natural killer (NK) and natural killer T (NKT) cells, and by antigen-presenting cells (APCs) like macrophages, dendritic cells and B-lymphocytes (Mosmann and Coffman, 1989; Bancroft et al., 1991; Flaishon et al., 2000; van Boxel-Dezaire and Stark, 2007). IFNγ is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IFNγ is also an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression. Although it was initially described for its antiviral properties, many studies have shown that IFNγ has significant inhibitory effects on fibroblast activation and proliferation and also reducing extracellular matrix production. For example, IFNγ is shown to have direct inhibitory effects on pancreatic stellate cells, reducing both cell proliferation and collagen synthesis *in vitro* (Baumert et al., 2006). Yao et al reported that IFNγ administration restored renal

histology and hemodynamics in an animal model of renal fibrosis after surgical reversal of hydronephrosis (Yao et al., 2011). Furthermore, IFNγ is known to inhibit TGFß-1 signaling to reduce fibrosis formation and improves the healing of lacerated skeletal muscle (Foster et al., 2003). The prominent antiproliferative and antifibrotic role of IFNγ has been well characterized by its function in inhibiting organ fibrosis and extracellular matrix production (Wen et al., 2004), thereby making it an interesting molecule for therapeutic use to target fibrosis. IFNγ has been used to treat fibrotic diseases in several organs, including hepatic fibrosis (Baroni et al., 1996), silicosis (Chen et al., 2005) and pancreatic fibrosis (Fitzner et al., 2007).

Substance P

The factor, which we will abbreviate as SubP, is a member of the tachykinin family. It is an 11 amino-acid neurotransmitter involved in nociception in the peripheral and central nervous systems. Substance P coexists with the excitatory neurotransmitter glutamate in primary afferents that respond to painful stimulation (Felipe et al., 1998). The sensory function of substance P is thought to be related to the transmission of pain information into the central nervous system (Zubrzycka and Janecka, 2000). In addition to its involvement in pain mechanisms, SubP has known pro-inflammatory effects on vasodilation and vascular permeablization (Harrison and Geppetti, 2001) as well as reparative responses including cell proliferation and angiogenesis (Fan et al., 1993). Its role in cell proliferation and angiogenesis is also shown in tendon tissue (Burssens et al., 2005; Carlsson et al., 2011). SubP is present and produced by many types of cells, including endothelial cells, eosinophils, macrophages, mast cells and tenocytes (Backman et al., 2011b). Studies have confirmed that SubP is

found both in rat flexor forelimb tendon tissues (Fedorczyk et al., 2010) and primary tenocytes derived from tendons (Backman et al., 2011b). Yet, its role in these tissues, independent of nociception, is not completely understand.

For example, SubP has been linked to fibrotic tissue events. Increased SubP has been detected in ligaments and tendons of patients with carpal tunnel syndrome (Ozturk et al., 2010). Tenocytes and fibroblasts in tendons collected from humans and animal models with overuse-MSDs also show increased SubP expression (Fedorczyk et al., 2010; Backman et al., 2011a; Backman et al., 2011b; Abdelmagid et al., 2012). Exogenously administered SubP promotes cellular proliferation in tendons during wound healing in rats (Burssens et al., 2005; Carlsson et al., 2011). Studies have also shown that exogenously administered SubP promotes human tenocyte proliferation (Backman et al., 2011b) as well as collagen remodeling *in vitro* (Fong et al., 2013) and tendinosis in an *in vivo* rabbit model of Achilles tendon overuse (Andersson et al., 2011). Increased SubP has been linked to increased collagen type I expression by dermal fibroblasts during the wound healing process (Chéret et al., 2014). Therefore, increased SubP appears to play a role in fibroblast proliferation and collagen production during wound healing. The mechanism by which it coordinates with TGFß-1 during inflammatory (Beinborn et al., 2010) and fibrotic tissue processes is unknown (Koon et al., 2010; Dehlin et al., 2013). It is also not known if SubP can effect CCN2 production and function independently of TGFß-1.

Aims and Goals

The long-term objective of this research is to examine the role of anti-fibrogenic protein, IFNγ and fibrogenic mediators, TGFß-1, CCN2 and SubP in fibrosis occurring in an *in vivo* rat model of upper extremity WMSD and in fibroblasts *in vitro*. This study will use histological and immunochemical techniques to access fibrogenic protein production *in vivo* and use cell-based assays to address the interactions between fibrosis-related mediators *in vitro*.

Specific aims:

Aim 1. To examine the effects of performing a high repetition high force (HRHF) handle-pulling task for up to 18 weeks on fibrotic responses in forearm muscles and surrounding ECM in a rat model of overuse injury.

Prior to this study, the inflammatory and fibrogenic processes had only been examined at shorter time periods (6-12 weeks of performance of a repetitive and forceful task). Since patients typically report and undergo treatment only when chronic WMSD symptoms are present, characterization of events occurring chronically will allow identification of mediators that can be targeted in future intervention studies. We hypothesized that inflammatory cytokine responses would resolve early, that fibrotic tissue responses (specifically, TGFß-1 and IFNγ) would increase significantly in flexor digitorum muscles and ECM in rats that are operantly performing a repetitive and forceful handle pulling task.

Aim 2. To determine if interactions between CCN2 and Substance P (SubP) are regulating dermal and tendon fibroblast proliferation and collagen production *in vitro*.

Little work has been done to understand the role of substance P in fibrosis,

particularly if it can induce fibroblast proliferation and collagen production via CCN2 signaling, independently of TGFß-1. We hypothesized that SubP either acts directly or indirectly through CCN2 or acts independently to increase fibroblast proliferation and extracellular matrix production in primary tendon fibroblasts (which will be termed as tenocytes) or rat dermal fibroblasts (RDF) *in vitro* (Fig. 1-6). This study will aid the understanding of the interactions of SubP and TGFß-1/CCN2 in tenocytes or RDF cell cultures.

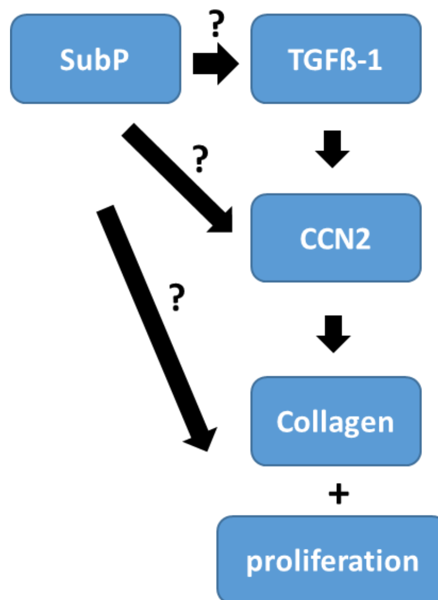

Figure 1-6. Schematic diagram showing pathways hypothesized to lead to fibroblast proliferation and collagen production *in vitro.* SubP could either act directly or indirectly through CCN2 or act independently to increase fibroblast proliferation and extracellular matrix production in primary tenocytes or rat dermal fibroblasts (RDF) *in vitro*.

CHAPTER 2

INCREASED TGFß-1 AND IFN-GAMMA ARE ASSOCIATED WITH FIBROSIS IN A RAT MODEL OF REPETITIVE OVERUSE INJURY

Overview

Key clinical features of cumulative trauma disorders include pain, muscle weakness, and tissue fibrosis, although the etiology is still under investigation. Here, we characterized the temporal pattern of fibrogenic processes occurring in forearm muscles of young adult, female rats performing an operant, high repetition high force (HRHF) reaching and grasping task for 6, 12, or 18 weeks. We evaluated the fibrogenic protein, TGFß-1, and the anti-fibrogenic protein, IFNγ. Both increased in and around flexor digitorum muscles and extracellular matrix in the mid-forearm. Thus, muscle fibrosis may be critical component of chronic work-related musculoskeletal disorders.

*Extracted from previously published work in which Zhao, Y contributed TGFß-1 and IFNγ data: Fisher PW, **Zhao Y**, Rico MC, Massicotte VS, Wade CK, Litvin J, Bove GM, Popoff SN, Barbe MF. Increased CTGF, Substance P and Tissue Fibrosis Are Associated with Sensorimotor Declines in a Rat Model of Repetitive Overuse injury. J Cell Commun Signal. 2015 Jan 24. [Epub ahead of print] DOI 10.1007/s12079-015-0263-0. PMID: 25617052

Introduction

The United States Occupational Safety and Health Administration (OSHA, 2014) estimates that work-related musculoskeletal disorders (WMSDs) account for over 600,000 injuries and illnesses (OSHA, 2014). These disorders are estimated at $20 billion a year in direct costs, and up to five times more in indirect costs for MSD-related workers' compensation, in addition to the substantial toll on affected workers who

develop significant difficulties in performing simple upper extremity tasks (OSHA, 2014). The incidence rate of occupation-related arm, wrist and hand injuries is 23% of all workplace injuries/illnesses and requires a median of 12 days away from work (BLS, 2013). Studies in humans with upper extremity WMSDs find evidence of inflammation, fibrosis and degeneration in tissues, changes thought to cause the concurrent sensorimotor dysfunction (Rempel and Diao, 2004; Carp et al., 2007; Heinemeier et al., 2007; Rechardt et al., 2011; Seher et al., 2011; Chikenji et al., 2014). However, underlying pathophysiological responses are incompletely understood, particularly those associated with chronic fibrotic pathologies.

Patients with chronic (>3 months) WMSDs show continued symptoms of pain and motor dysfunction, yet an absence of inflammatory markers. Instead, these latter patients have increased tissue fibrosis and fibrogenic markers, such as transforming growth factor beta 1 (TGFß-1) (Freeland et al., 2002; Ihn, 2008; Beinborn et al., 2010; Gao et al., 2013; Chikenji et al., 2014), although serum biomarkers of fibrosis in relationship to WMSDs are still under investigation.

We have developed a unique rodent model of operant repetitive reaching and grasping in which the performance of a reaching and handle-pulling task causes injury and inflammation, followed by tissue fibrosis (Fedorczyk et al., 2010; Abdelmagid et al., 2012; Gao et al., 2013). We observed exposure-dependent declines in sensorimotor function after short-term performance (≤3 months) of these tasks, with a high repetition high force (HRHF) task inducing the greatest dysfunction (Fedorczyk et al., 2010; Abdelmagid et al., 2012). Short-term sensorimotor declines were associated with duration- and force-dependent increases in tissue inflammation and fibrosis (Clark et al.,

2004; Elliott et al., 2009; Fedorczyk et al., 2010; Kietrys et al., 2011; Abdelmagid et al., 2012; Barbe et al., 2013a; Gao et al., 2013). Unfortunately, we were not able to tease out if tissue inflammation or fibrosis (or both) were contributing to functional declines in these past studies of ≤ 3 months, due to concurrent inflammatory and fibrotic tissue responses.

Therefore, our goal here was to examine the effects of performing a high repetition high force (HRHF) handle-pulling task for up to 18 weeks on fibrosis-related proteins production in forearm muscles and surrounding ECM, specifically TGFß-1 and IFNγ, and their sources in a rat model of long term upper extremity overuse injury, using western blotting and immunofluorescence methods. We hypothesized that inflammatory cytokine responses would resolve early, that fibrotic tissue responses (specifically, TGFß-1 and IFNγ) would increase significantly in flexor digitorum muscles and ECM with prolonged performance of a high demand repetitive task. This author's (YZ) part of the project was to perform western blot analyses of IFNγ and TGFß-1, and to aid in the immunofluorescence quantification of IFNγ in tissues with long-term HRHF task.

Methods

Subjects

The Temple University Institutional Animal Care and Use Committee approved all experiments in compliance with NIH guidelines for the care and use of laboratory animals. Ninety-five young adult, female Sprague-Dawley rats (3 mo of age at onset of experiments) were used. Adult female rats were used in this study because: (1) Human

females have a higher incidence of work-related musculoskeletal disorders than males (Barbe et al., 2008); and (2) for comparison to data from our past studies on female rats using this model.

Rats were randomly divided into 4 groups: age-matched normal controls (NC, n=15): age- and weight-matched food restricted controls (FRC, n=23); age-matched trained-only rats that underwent an initial training (trained to high-force, TRHF, n=15) and then euthanized after training; and age-matched rats that were trained before performing the high-repetition, high-force task (HRHF) task for 3 weeks (n=6), 6 weeks (n=8), 12 weeks (n=12) or 18 weeks (n=18) before euthanasia (total number of HRHF rats = 42). Rats were housed in a central animal facility in separate cages with a 12-hour light:dark cycle and free access to water. Rats were weighed weekly and their food adjusted to maintain ± 95% body weight of age-matched controls to avoid catabolic tissue changes that might occur with greater weight loss, and to avoid confounds of obesity (rats tend to work hard for the banana-flavored food pellets used as food reward). All rats were inspected weekly and again post-mortem for presence of illness or tumors to reduce confounders for serum cytokine increases (none were observed). To further reduce illness-related confounders, additional sentinel rats were examined for presence of viral infections or other illnesses as part of regular veterinary care (none were detected).

Behavioral Apparatuses, Training and Task Regimen

Sixteen custom-designed behavioral apparatuses were used for these experiments, as previously described and depicted (Barbe et al., 2008). Briefly, animals reached through a shoulder height portal and isometrically pulled a force handle attached to a force

transducer with a load cell (Futek Advanced Sensor Technology, Irvine, CA) located outside the chamber wall. The load cell was interfaced with custom Force-Lever software (version 1.03.02, Med Associates, St. Albans, VT). Auditory and light indicators cued the reaching rate (defined below). If reach and force criteria (defined below) were met within a 5 second cueing period, a 45 mg food pellet was dispensed into a food trough (Fig. 2-1).

The trained-only (TRHF) and HRHF rats underwent an initial training period for 5 weeks in which they learned the task, as previously described (Elliott et al., 2009). Briefly, all but NC rats were initially food-restricted for 7 days to no more than 10-15% less than their naive weight, and the weight of age-matched rats with free access to food, to initiate interest in food reward pellets. After that week, they were given extra rat chow to gain weight quickly back to only 5% less than age-matched normal control rats. Rats were weighed weekly, and allowed to gain weight during the study as they were young adult rats (Elliott et al., 2010). The food-restricted rats trained to learn the HRHF reaching and handle-pulling tasks during a 5-week period of 10 min/day, 5 days/wk, in which they ramped upwards from naive towards the HRHF task level (see below). Trained rats reached the HRHF level only during their last week of training. After training, rats were randomly divided into TRHF and HRHF rats. TRHF rats were euthanized at this point to determine the effect of training. The remaining trained rats went on to perform the HRHF task.

After the initial training period, a point equal to week 0 of the HRHF task, 30 rats began the HRHF task regimen for 2 hrs/day, 3 days/wk for up to 18 weeks. The task was divided into 4, 0.5 hr sessions separated by 1.5 hrs in order to avoid satiation. HRHF rats were cued to reach at a rate of 8 reaches/min and to grasp the force handle at a target

force effort of 55% ± 5% (which equals 120 to 128 grams, and 1.02 to 1.12 Newtons). HRHF rats had to grasp the force handle and exert an isometric pull at the target level for at least 50 ms to receive a food reward, as described previously (Barbe et al., 2008). Rats were allowed to use their preferred limb to reach (the ‘‘reach’’ limb), and data from this limb only is reported.

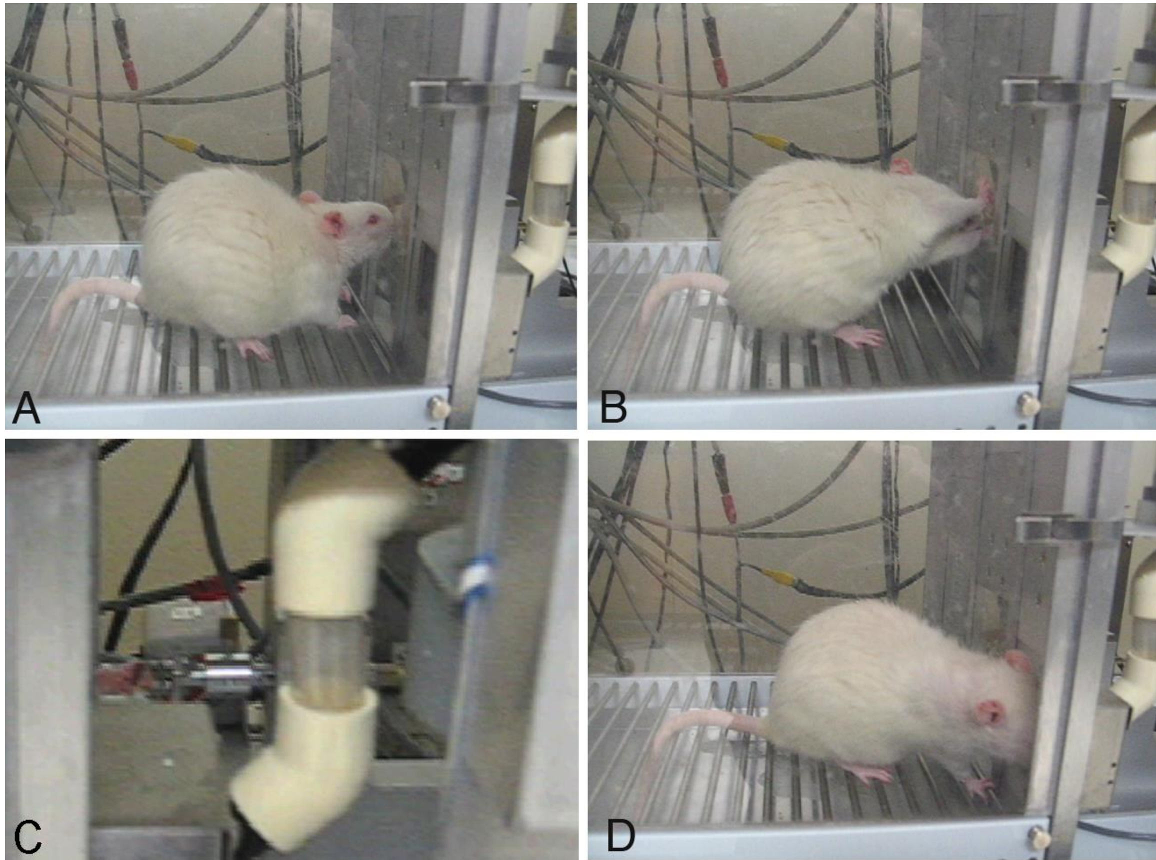

Fig. 2-1. Photographs of a rat performing the HRHF repetitive reaching task. (A) Rat awaits auditory stimulus with snout in portal. (B) Rat reaches for force handle with left forepaw; right forepaw used for postural support. (C) Viewed from top, rat grasps and isometrically pulls force handle attached to force transducer, until predetermined force threshold is reached and held for at least 50 ms. (D) Rat retrieves foot pellet reward by mouth from food trough. *(From "The interaction of force and repetition on musculoskeletal and neural tissue responses and sensorimotor behavior in a rat model of work-related musculoskeletal disorders." BMC musculoskeletal disorders 14(1): 303. Figure used with permission from Copyright Center Clearance.)*

Tissue Biochemical Assays for TGFß-1 and IFNγ

Following euthanasia and after serum collection, flexor digitorum muscles and tendons were collected from the distal forearm region of the preferred reach limb. Muscles were homogenized as previously described (Kietrys et al., 2012) from: NC (n=4), FRC (n=8), TRHF (n=4), and HRHF rats that performed the task for 6 (n=4), 12 (n=3) or 18 weeks (n=8). Tendons were homogenized similarly for NC (n=3) and 18 week HRHF rats (n=5). Muscle homogenates were also assayed via Western Blot for TGFß-1 (MAB240, 1:500, R&D Systems, Minneapolis, MN), and IFNγ (PA1-24782, 1:500, Thermo Scientific). Densitometry was performed using myImageAnalysis version 1.1 (Thermo Fisher Scientific). GAPDH was used as a loading control (glyceraldehyde-3-phosphate dehydrogenase; AM4300, Invitrogen). Western blots were repeated at least three times for TGFß-1 and IFNγ.

Immunohistochemical Analyses of TGFß-1 and IFNγ

Forelimb tissues that were not homogenized for biochemical analysis were used for histological analysis from: FRC (n=12) and HRHF rats that performed the task for 3 (n=6), 6 (n=4), 12 (n=4) or 18 weeks (n=8). Following euthanasia and after serum collection, animals were perfused transcardially with 4% buffered paraformaldehyde. Forearm musculotendinous tissues were collected and sectioned longitudinally, as described previously (Fedorczyk et al., 2010; Abdelmagid et al., 2012). Sections were immunostained in batched sets by the same individual for TGFß-1 and IFNγ, using previously described methods (Fedorczyk et al., 2010; Gao et al., 2013), and the following antibodies: mouse monoclonal anti-TGFß-1 at 1:500 dilution (MAB240, R&D Systems, Minneapolis, MN) and goat polyclonal anti- IFNγ (PA1-24782, 1:500, Thermo

Scientific, Rockford, IL). Briefly, after a 0.5% pepsin antigen retrieval step for 15 minutes at room temperature, sections were incubated for 20 minutes in the appropriate blocking serum for each antibody, and then were incubated with the primary antibody at the listed dilution for overnight at 4°C. This was followed by incubation with appropriate secondary antibodies that were AffiniPure $F(ab')_2$ fragments, preabsorbed to reduce non-specific cross-reactivity with rat antigens, and conjugated to green or red fluorescent cyanine dyes (Cy2, DyLight 488, Cy3, or DyLight 594) (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:100 each for 2 hours at room temperature.

The specificities of the TGFß-1 antibody and IFNγ antibody are shown in a western blot that is part of this study. Preabsorption controls were also performed to demonstrate if the antibodies bound specifically to the antigen of interest using: TGFß-1 human recombinant protein (GF111, Millipore, Temecula, CA). A ten-fold excess of purified protein or peptide was pre-incubated with the matching antibody overnight at 4°C, the mixture centrifuged, and then the pre-absorbed antibody supernatant was incubated with the tissues (after pepsin and goat serum treatment) similarly to that described above before washing and incubation with secondary antibodies. No labeling was observed in the tissues for any pre-absorbed antibody (Fig. 2-2). We also performed no primary antibody controls in which serum was substituted for the primary antibody, followed by secondary antibodies; no labeling was observed as a result of incubation of tissues with serum and then secondary antibodies alone.

Quantification of TGFß-1 and IFNγ

The percent area of immunostaining for TGFß-1 and IFNγ was quantified in batched sets in the epimysium region and in the ECM adjacent to the flexor digitorum muscles

using an image analysis system (BioQuant Osteo, BioQuant, Nashville, TN), using previously described methods (Al-Shatti et al., 2005; Fedorczyk et al., 2010; Gao et al., 2013).

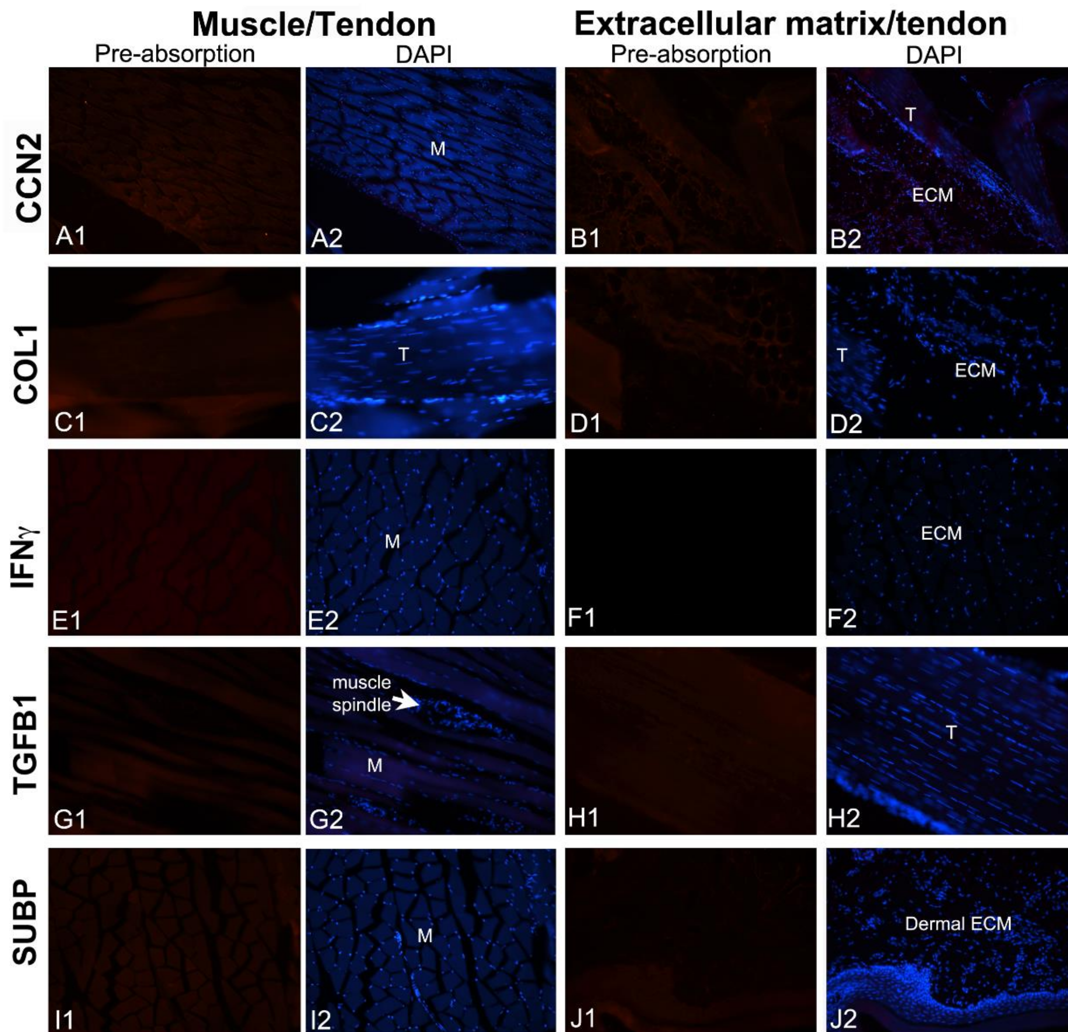

Fig. 2-2. Pre-absorption control staining demonstrating antibody specificity in flexor digitorum muscle (M), tendon (T) or extracellular matrix tissues from HRHF rats. DAPI (blue) used as nuclear stain. TGFß-1 antibody staining after preabsorption with TGFß-1 rat recombinant protein *(From Fisher P, et al. 2015. Increased CCN2, substance P and tissue fibrosis are associated with sensorimotor declines in a rat model of repetitive overuse injury. J Cell Commun Signal: 1-18. Figure used with permission from Copyright Clearance Center.)*

Statistical Analyses

GraphPad PRISM v.6.02 was used for the statistical analyses. All data are expressed as mean ± SEM. P values of < 0.05 were considered significant for all comparisons. During the post-hoc ANOVA analyses for between group differences, the Bonferroni correction method was used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data. In accordance with this method, an adjustment was made to *p* values by the analysis program used (GraphPad PRISM) by dividing the critical p value ($\alpha = 0.05$) by the number of comparisons made, thus increasing the stringency of the analysis. For succinctness, specifics of ANOVA and significant posthoc findings are reported in the figure panels.

Results

Fibrogenic protein TGFß-1 and anti-fibrogenic protein IFNγ increase in tissues with long-term HRHF task

IFNγ immunoexpression was increased in small cells on the perimeter of myofibers of 18-week HRHF rats, compared to FRC rats (Fig. 2-3B versus Fig. 2-3A). As shown in the western blot image located to the right of Fig. 2-3B, the anti-rat IFNγ antibody recognized a band at the relative molecular weight of 17 kDa. CCN2 immunostaining was also increased in 18-week HRHF rat muscles in small cells on the perimeter of myofibers and endomysium, compared to the FRC rats (Fig. 2-3B versus Fig. 2-3A).

TGFß-1 immunoexpression also increased in small cells on the perimeter of myofibers and in flexor digitorum tenocytes of 18-week HRHF rats, compared to FRC

rats (Fig. 2-3D and F, versus Fig. 2-3 C and E). The TGFß-1 immunostained tenocytes in the tendons of the 18-week HRHF rats were enlarged and rounded in appearance, compared to the elongated tenocytes seen in the FRC rats (Fig. 2-3E and F). As shown in the western blot image located to the right of Fig. 2-3D, the anti-rat TGFß-1 antibody recognized a band at the relative molecular weight of 50 kDa.

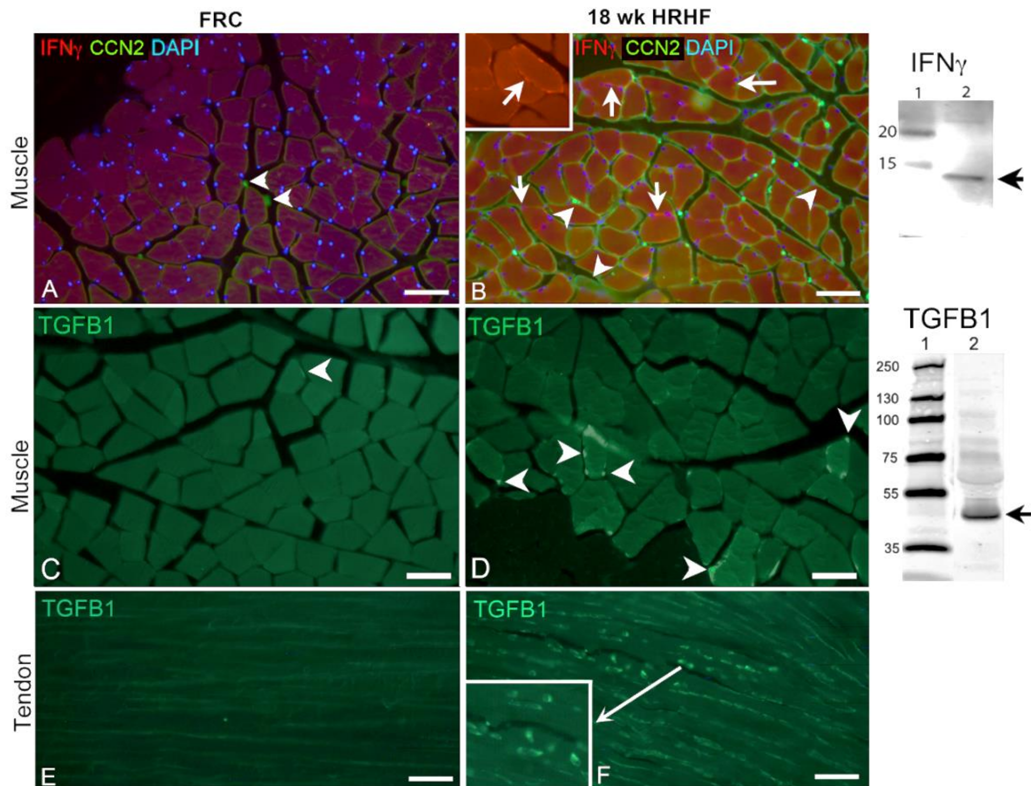

Figure 2-3. IFNγ, CCN2, and TGFß-1 immunostaining in flexor digitorum muscles and tendons of FRC and 18-week task rats. DAPI (blue) used as nuclear stain. (A, B) IFNγ (red) and CCN2 (green) immunostaining in muscle of FRC and 18 week HRHF rats. Inset in B: IFNγ immunostaining in small cells on myofiber perimeter. Arrows indicate examples of IFNγ stained cells; arrowheads indicate CCN2 deposition around myofiber perimeter. (C, D) TGFß-1 (green) immunostaining in muscle of FRC and 18 week HRHF rats. (E, F) TGFß-1 in tenocytes of FRC and 18 week HRHF rat tendons (inset shows enlargement of area indicated with arrow). The Western blots for IFNγ and TGFß-1 show the bands recognized by the antibodies used for IHC. Scale bar = 50 micrometers *(From Fisher P, et al. 2015. Increased CCN2, substance P and tissue fibrosis are associated with sensorimotor declines in a rat model of repetitive overuse injury. J Cell Commun Signal: 1-18.* Figure *used with permission from Copyright Clearance Center.)*

The endomysium differences were confirmed by quantification of TGFß-1 and IFNγ immunostaining (Table 2-1). There was a significant increase of TGFß-1 and IFNγ immunostaing in the endomysium of 18-week HRHF rat muscles, compared to FRC rats. TGFß-1 or IFNγ immunostaing was not evident in FRC rat muscles (Fig. 2-3A, C, E; Table 2-1).

Table 2-1. Quantification of Immunofluorescence (% area with immunostaining; Mean ± SEM)

Analyte	FRC rats (n=3-13)	3 wk HRHF rats (n=4)	6 wk HRHF rats (n=4)	12 wk HRHF rats (n=4)	18 wk HRHF rats (n=3-8)
			Flexor Digitorum Muscle - Endomysium		
IFN-gamma	0.75 ± 0.48	n.t.	n.t.	n.t.	25.50 ± 8.15*
TGFß-1	1.04 ± 0.33	2.21 ±0.71	6.28 ± 0.92	21.79 ± 1.59**	16.38 ± 2.71**

* and **: p<0.05 and p<0.01, compared to FRC rats; n.t. = not tested.

Discussion

We found, as hypothesized, that the tissue inflammatory cytokine responses resolved early, and that the fibrogenic tissue protein TGFß-1 and anti-fibrogenic protein IFNγ, increased significantly in and around flexor digitorum muscles and extracellular matrix with prolonged performance of a HRHF task. An observed increase in serum TGFß-1 is supportive of it serving as a potential biomarker of tissue fibrosis and reduced function that occurs as a consequence of overuse.

Specifically, we observed an increase in a key fibrogenic related protein, TGFß-1, in the extracellular matrix and muscles of rats that had performed the HRHF task for 18 weeks. TGFß-1 immunostained cells within the muscles were likely fibroblasts, while

those in tendons were tenocytes (a subset of fibroblasts specific to tendons). Other groups have shown that fibrogenic proteins increase in fibroblasts and tenocytes under conditions of tissue overload or injury, such as TGFß-1. CCN2, IL-1ß, IL-6, and IL-8, insulin-like growth factor I (IGF-I), fibroblast growth factors (FGF), NO, prostaglandins, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) (Kjaer, 2004; Nakama et al., 2006; Smith et al., 2007; Elliott et al., 2010; Barbe et al., 2013a).

The anti-fibrotic agent, IFNγ (Ziesche et al., 1999; Foster et al., 2003; Diaz et al., 2012), was increased in serum and muscles at 18 weeks of HRHF task performance. IFNγ is an anti-fibrotic cytokine known to inhibit TGFß-1 signaling (Leask and Abraham, 2004). Foster et al showed that the anti-fibrotic agents decorin and IFNγ can decrease fibrotic deposits in a mouse laceration injury model, and that IFNγ administration can improve muscle function (Fukushima et al., 2001; Foster et al., 2003). We suggest that the increased IFNγ was an attempt by HRHF rats to control a fibrotic tissue response.

Clinically, TGFß-1 is increased in tenosynovium and subsynovial connective tissue of carpal tunnel syndrome patients (Chikenji et al., 2014). Studies have shown that anti-TGF-ß1 treatment can inhibit fibrosis development. Cohn et al reported that Losartan, which has anti-TGFß-1 effect, improved muscle morphology and enhanced muscle regeneration in a mouse model of Marfan syndrome (Cohn et al., 2007). Bedair et al demonstrated that losartan can reduce muscle fibrosis, increase the number of regenerating myofibers, and enhance the physiological function of injured muscle through the inhibition of TGF-ß1 (Bedair et al., 2008). Terada et al further showed that blocking TGF-ß1 by administering losartan in addition to platelet-rich plasma (PRP)

reduced fibrosis development in injured muscle and further enhance the functional recovery in a mouse model of muscle contusions (Terada et al., 2013). Since both PRP and losartan is clinically available, this combined treatment could be readily applied clinically for the treatment of muscle injuries or fibrosis.

Although not the focus of my contributions to this manuscript, CCN2 also increased with 18 weeks of HRHF performance. Increased CCN2 has been linked to the pathogenesis of tissue fibrosis (Tzortzaki et al., 2007; Elliott et al., 2010; Seher et al., 2011; Balayssac et al., 2014). It is the downstream mediator of TGFß-1 (Grotendorst, 1997; Song et al., 2007; Barbe et al., 2008; Fedorczyk et al., 2010), and TGFß-1-induced CCN2 expression leads to fibroblast proliferation and extracellular matrix deposition (Grotendorst, 1997).

In conclusion, these findings show that high demand tasks can induce tissue fibrotic changes if the work is performed for long periods of time. The increased tissue fibrotic proteins suggest that fibrogenic processes increase with continued overuse-type tasks. Our results showing that TGFß-1 is increased in serum too, suggest that TGFß-1 may serve as a serum biomarker of fibrosis occurring as a consequence of WMSDs.

CHAPTER 3

SUBSTANCE P INCREASES CCN2 INDEPENDENTLY OF TGFß-1 IN RAT TENOCYTES AND DERMAL FIBROBLASTS IN VITRO

Overview

Little work has been done to understand the role of substance P in fibrosis, particularly if it can induce fibrosis via CCN2 production and signaling, independent of TGFß-1. We hypothesized that SubP either acts directly or indirectly through CCN2 or acts independently to increase fibroblast proliferation and extracellular matrix production in primary tendon fibroblasts (which will be termed as tenocytes) or rat dermal fibroblasts (RDF) cells *in vitro*. This study will help us understand the interactions of SubP and TGFß-1/CCN2 in tenocytes or RDF cell cultures.

Introduction

Tendinosis is a chronic condition observed in patients with various musculoskeletal disorders and it is also one of the biggest outcomes of overuse-musculoskeletal diseases (MSDs) (BLS, 2013). The mechanisms behind tendinosis are incompletely understood, although changes in fibrosis and collagen reorganization are hallmark features of this condition. Several studies have identified proteins involved in fibrosis in tendons and tenocytes, including transforming growth factor beta (TGFß-1), CCN2 (formerly known as connective tissue growth factor, or CTGF), and substance P (SubP) for their effects on collagen production. TGFß-1 has been shown to be a key

regulator of collagen production in multiple tissue and cell types, including tendon and tendon fibroblasts (Kjaer et al., 2009; Chikenji et al., 2014; Chen et al., 2015). SubP and CCN2 are also upregulated under chronic overload conditions in tenocytes collected from humans with MSDs (Backman et al., 2011b; Fong et al., 2013; Chikenji et al., 2014) and in muscle and tendon tissues from animal models of overuse-MSDs, including our own rat model of repetitive reaching and grasping (Nakama et al., 2006; Fedorczyk et al., 2010; Andersson et al., 2011; Backman et al., 2011b; Abdelmagid et al., 2012; Fong et al., 2013; Gao et al., 2013; Fisher et al., 2015).

In this study, we attempt to understand the interplay of these three pro-fibrotic molecules, TGFß-1, CCN2 and SubP. Several studies report that CCN2 is a downstream mediator of TGFß-1 signaling in osteoblasts, dermal fibroblasts, and myoblasts, both proteins functioning through SMAD pathways (Leask, 2004; Arnott et al., 2007; Sobral et al., 2007; Arnott et al., 2008). TGFß-1 and SubP have also been reported to work in concert, either by TGFß-1 mediated SubP receptor regulation (Beinborn et al., 2010), transcriptional modulation of each other (Lai et al., 2003; Yaraee and Ghazanfari, 2009; Kant et al., 2013), or through cooperative signaling of these two molecules leading to enhanced fibroblast proliferation or collagen production (Katayama and Nishioka, 1997; Koon et al., 2010).

The importance of neurochemical modulators such as SubP in fibrosis have only emerged in the last decade. SubP is primarily known as a neuropeptide involved in pain transmission. More recently however, SubP has been shown to be involved in both inflammation via its production by macrophages, eosinophils, mast cells, lymphocytes, and dendritic cells, and in fibrotic disorders of various tissue types such as lung and

intestines [(Yaraee and Ghazanfari, 2009; Koon et al., 2010), reviewed in (O'Connor et al., 2004; Steinhoff et al., 2014)]. In terms of overuse-MSDs, SubP has been implicated in the pathogenesis of tendinosis in patients (Riley, 2008), as well as in *in vivo* Achilles loading models in rats and rabbits (Messner et al., 1999; Backman et al., 2011a; Backman et al., 2011b).

In our own model of overuse-MSDs, we have identified increased SubP, along with collagen, TGFß-1, and CCN2, in the flexor digitorum tendons of rats performing a repetitive reaching task (Fedorczyk et al., 2010; Gao et al., 2013; Fisher et al., 2015). Our goal here was to use *in vitro* experiments on isolated tenocytes to further elucidate the role of SubP on CCN2 and collagen production. It is still unclear whether SubP directly causes fibrotic responses in tenocytes (CCN2 production, cell proliferation, and collagen deposition), or whether these fibrotic responses require TGFß-1 as an intermediate or co-signaling molecule. We hypothesized that SubP may act directly through CCN2 to increase fibroblast proliferation and extracellular matrix production. It is possible that SubP and TGFß-1 may act in an additive or synergistic fashion to promote fibroblast proliferation and collagen production.

In this study, we hoped to confirm the findings reported above from other studies of cultured tenocytes in our isolated rat flexor digitorum tenocytes. In addition to tenocytes, RDF cells were used in these experiments since these cells are known to produce collagen in response to TGFß-1 (Howard et al., 2012), and could thus serve as a positive control. Specifically, these studies were intended to demonstrate that: 1) SubP treatment induces TGFß-1 release in primary rat flexor digitorum tenocytes, 2) SubP treatment induces cell proliferation in our tenocyte cultures, and 3) that SubP treatment

induces markers of fibrosis, namely CCN2 and collagen. Since both SubP and CCN2 have been shown to stimulate fibrotic responses in fibroblasts, we hypothesized that SubP may act directly through CCN2 to increase fibroblast proliferation and extracellular matrix production. We also sought to determine if SubP and TGFß-1 act in an additive or synergistic fashion to promote fibroblast proliferation and collagen production in cultured tenocytes and/or RDF cells under unloaded conditions. These studies are expected to address whether CCN2 acts as an essential mediator of the pro-fibrotic effects of SubP, and whether successful anti-fibrotic therapies for overuse-MSDs need to block one or more specific signaling pathways to achieve the most efficacious outcome.

Methods

Isolation of tendon cells from rat subjects. Samples of rat flexor digitorum tendon tissue were obtained under sterile conditions from normal young adult female Sprague-Dawley rats. The Temple University Institutional Animal Care and Use Committee approved all experiments in compliance with NIH guidelines for the care and use of laboratory animals.

Primary culture of rat flexor digitorum tendon cells. The tendon cell culture was performed as previously reported by Backman's laboratory, in which tenocytes were collected from human Achilles tendons of healthy donors (Backman et al., 2011b). In our study, rat flexor digitorum tendon tissue were washed with sterile Hank's Balanced Salt Solution (HBSS; Invitrogen; 14170) and carefully dissected and minced using scissors in HBSS. The minced fragments were enzymatically digested at 37°C using collagenase (2mg/ml, Clostridopeptidase A, C-0130, Sigma, Saint Louis, MO) diluted in D-MEM (Dulbecco's Modified Eagle Medium, 10-013-CV, Corning, Manassas, VA). The

digested product was then centrifuged at 800 x g for 5 min, the supernatant was discarded, and the pellet was re-suspended and cultured in D-MEM supplemented with 10% fetal bovine serum (FBS; S11150, Atlanta Biologicals, Lawrenceville, GA), 1% penicillin/streptomycin and 2% L-Glutamate (25-005-CI, Sigma corning, Saint Louis, MO) at 37°C in a humidified atmosphere of 5% CO_2 in air. The media was changed every third day. Confluent cells were harvested using 0.05% trypsin with EDTA and re-suspended in medium and seeded at a 1:3 ratio. These cultures were expanded and cells from passages 3 to 6 were used in this study. In all experiments, the concentration of serum was reduced from 10% to 1% for 24 h prior to cell treatment to eliminate any unwanted effects of the serum. The serum-deprived cells appeared healthy and intact.

Rat dermal fibroblasts. Rat dermal fibroblasts (RDF) purchased from Cell Applications and initially cultured in the provided fibroblast growth medium (Cell Applications, Inc; R115-500). After passaging, the cells were maintained in D-MEM (Corning; 10-013-CV) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin/streptomycin and 2% L-Glutamate (Sigma corning; 25-005-CI) at 37°C in a humidified atmosphere of 5% CO_2 in air. In all experiments, the concentration of serum was reduced from 10% to 1% for 24 h prior to cell treatment to eliminate any unwanted effects of the serum. The serum-deprived cells appeared healthy and intact.

SubP and TGFß-1 treatment of cultured fibroblast cells. After 24 h serum deprivation, RDF cells or isolated tenocytes were treated with either 100 nM of SubP (1156, Tocris), 10ng/ml TGFß-1 (GF111, EMD Millipore, Temecula, CA) alone or their combination. For collagen deposition assessment, cells were treated in the presence of

ascorbic acid (20 µg/mL). For proliferation and CCN2 expression experiments, further 1:10 or 1:4 dilutions were made in the reconstitution buffer (PBS + 1% BSA) to evaluate a dose response. The unit of TGFß-1 (ng/mL) or SubP (nM) used was reported as in previous publications (Arnott et al., 2007; Beinborn et al., 2010; Backman et al., 2011b). Cells were also treated with TGFß-1 and SubP in combination, in the concentrations indicated in the figures and legends, to explore possible additive effects of these agonists. During this treatment period of up to 72 h, the concentration of serum was maintained at serum deprivation level (1%); the cells continued to appear healthy and intact.

Immunocytochemical Analyses and Quantification

Immunocytochemistry for vimentin and tenomodulin in chamber slide cultures. 10,000 cells were seeded per well on 8-well chamber slides (177445, Lab-Tek™) overnight before being processed for vimentin and tenomodulin. Cells were fixed in 4% paraformaldehyde for 10 min followed by 4 × 3 min wash in PBS. 0.1% Triton X-100 in PBS was applied for 15 min to ensure cell permeabilization. A mouse monoclonal anti-vimentin antibody (1:100; M0725, Dako, Glostrup, Denmark) and a goat polyclonal anti-tenomodulin antibody (1:100; sc-49325, Santa Cruz, CA) were used on fixed cell cultures. After blocking with 5% donkey normal serum for 15 min, the chamber slides were incubated with the primary antibody for 60 min at 37°C. After additional washing and blocking in 5% donkey serum for 15 min, the secondary antibody, TRITC donkey anti-mouse (1:2000; 610-709-124, Rockland Immunochemicals, Limerick, PA) or FITC donkey anti-goat (1:2000; 605-702-002, Rockland Immunochemicals, Limerick, PA) was incubated for 30 min at 37°C before washing and mounting in SlowFade Diamond with DAPI (S36968, Life Technologies, Grand Island, NY) for fluorescence imaging.

Immunocytochemistry for CCN2 in chamber slide cultures.

For immunocytochemical analyses of the tenocytes or RDF cells, 2,000 cells per well were seeded on 8-well chamber slides (177445, Lab-Tek™) overnight and then serum deprived for 24 h prior to treatment. Cells were treated with a standard concentration of 100 nM of SubP (1156, Tocris), 10 ng/mL of TGFß-1 (GF111, EMD Millipore) alone or their combination for a 48 h period. Fixation of cells was performed with 4% paraformaldehyde for 20 min and thereafter washed with phosphate buffered saline (PBS) + 0.05% Tween-20 and permeabilized with 0.1% Triton X in PBS for 15 min. after blocking in 3% Bovine serum albumin (BSA; Fisher Scientific, BP1605-100) for 30 min at room temperature, the cells were then incubated with a CCN2 goat antibody at a concentration of 1:50 (Santa Cruz, sc-14939) at 4°C overnight. Afterwards cells were washed and incubated with a secondary antibody, Cy2 donkey anti-goat (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500 each for 60 min at room temperature. Nuclei counterstaining was performed with DAPI for 5 min at room temperature. Cells were washed with PBS + 0.05% Tween-20. The experiment was repeated three times.

Quantitative analyses of CCN2 immunocytochemistry findings. To quantify the changes in CCN2 immunoexpression per cell, immunofluorescent stained chamber slides were analyzed using a microscope (Nikon) interfaced with an analog camera and an image analysis system (BioQuant Osteo, BioQuant, Nashville, TN). All assessments and image analyses were carried out in a blinded fashion. The microscope's light intensity was maintained at a constant level to ensure the background values were similar for each acquired image. Likewise, the f-stop for the camera was maintained at a constant level for

each acquired image. Measurements were made only within the cytoplasm of each cell using the circle Region of Interest Option (ROI) of the BioQuant software. The circle was 3.85×10^{-9} m^2 in size. The Videocount Area Array option of the BioQuant software was also utilized for these measurements. Videocount area is defined as the number of pixels in a field that meet a user-defined color threshold of green immunofluorescence staining multiplied by the area of a pixel at the selected magnification (20x in our analyses). The CCN2 green staining threshold values were saved in a subprogram created in BioQuant for this set of analyses, and then applied for each experiment for consistent auto-measurement of the immunostained chamber slides. Gain and exposure were standardized to allow for the cleanest image with fast, short exposure times and remained constant for the entire fluorescent quantification. The number of pixels at or above the defined threshold within the circled area (3.85×10^{-9} m^2) is reported. For tenocytes, a total number of 200 cells/group collected from three separate experiments were counted. Group means plus standard error of the mean (SEM) of the mean area (n =3) were plotted.

Cell Proliferation

Cell number was determined using the CyQUANT NF Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, cells were plated at 2,000 cells/well in a 96 well plate (Falcon) in D-MEM/10% FBS, serum deprived for 24 h and treated with SubP (Tocris; 1156), TGFß-1(EMD Millipore; GF111) alone or their combination at a range of doses. FGF-2 (Fibroblast growth factor-2; Sigma, SRP 4038) was used as a positive control. A control without treatment was also included. After 72 hours, media was aspirated and replaced with DNA binding dye

solution. Cells were incubated at 37°C for 30 min and samples were measured using a Wallac 1420 fluorometer. Proliferation data was normalized using the GraphPad PRISM software to compare data across experiments, with 100% set to 10ng/mL of FGF-2 and 0% defined as vehicle control (PBS + 1% BSA). Each set of CyQUANT experiments were repeated three times, in duplicate for each experimental condition.

CyQUANT proliferation was also verified one time each with the BrdU incorporation and Alamar Blue methods. For each, RDF or tenocytes were seeded at 2000 cells/well in 96 well plate in DMEM + 10% FBS and incubated overnight at 37°C to allow for cell adherence. The following day, the media was changed to 1% FBS and cells were treated with a dose range of SubP, TGFß-1, the combination of SubP + TGFß-1, or fibroblast growth factor-2 (as a positive control) at the concentrations indicated in the figures. The cells were incubated for ~72 hrs for each assay. For BrdU incorporation, the assay was performed according to the manufacturer's instructions (11647229001, Roche Diagnostics, Indianapolis, IN). Briefly, bromodeoxyuridine was added to the cells for 6 h prior to the 72 h endpoint, and the cells were incubated at 37°C to allow for DNA incorporation of the BrdU. After this period, the media was removed and the cell were fixed with the provided fixation buffer. Anti-BrdU-peroxidase antibody was used to detect incorporated BrdU a relative indicator of cell division. Absorbance was measured at 450nm after incubation with peroxidase substrate. For alamar blue measurement, alamar blue (BUF012B, Bio-Rad AbD Serotec, Raleigh, NC) was added to the cells in culture for four hours before the 72 h endpoint and the plate were re-incubated at 37°C. The color change in alamar blue dye was measured at absorbance 570nm.

Biochemical analyses

CCN2 In-Cell Westerns. Rat tenocytes or dermal fibroblasts were seeded at 10,000 cells/well and stimulated with either SubP, TGFß-1, or their combination. Media was changed at 24h, the cells were retreated, and CCN2 analysis was performed at 48 hours. For protein analysis, cells were washed three times with PBS and fixed in 95% ethanol for 20 minutes at room temperature. Plates were blocked with Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, Nebraska) for 1 hour at room temperature. Anti-CCN2 antibody (SC-14939, Santa Cruz Biotechnology, Santa Cruz, CA) was added at 1:2000 dilution and the plate was incubated at room temperature for 2 hours with gentle shaking. After two hours, the plate was washed four times with PBS plus 0.05% Tween-20. Anti-goat IRDye800 (LI-COR, diluted 1:800 in Odyssey blocking buffer) was added to each well. CellTag 700 was added at the same time as a secondary antibody for cell normalization (diluted 1:500). The plate(s) were incubated for 1 hour at room temperature, washed four times with PBS plus 0.05% Tween-20, and detection was performed using the Odyssey Imager.

Western Blotting. Cells were lysed at 4°C in protein extraction buffer (RIPA buffer, N653, AMRESCO Inc, Solon, OH) freshly supplemented with a protease inhibitor cocktail at a dilution of 1:100 (P8340, Sigma, St. Louis, MO). Protein concentration was determined by using Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific, Rockford, lL) according to manufacturer’s instructions. Total protein (50µg) from each sample was diluted in a Laemmli sample buffer (Bio-Rad; 161-0747) and boiled for 5 min before resolving by 10% SDS–PAGE. Protein samples were electroblotted to a

nitrocellulose membrane at 100 V for 75 min. After membranes were blocked in 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBS-T) for 1 h, blots were incubated overnight at 4°C with a primary antibody against CCN2 (1:100, SC-14939, Santa Cruz Biotechnology, Santa Cruz, CA), or TGFß-1 (1:200, SC-146, Santa Cruz Biotechnology, Santa Cruz, CA). After three washes of the membrane in TBS-T for 5 minutes, donkey anti-goat-IRDye® 680 (1:5000; #926-32224, Li-Cor) or donkey anti-mouse-IRDye680LT (1:5000; #926-32212, Li-Cor) was incubated for 60 min at room temperature. The membrane was washed as before and bands were detected using the Li-Cor Odyssey Infrared Imaging System. Blots were concurrently probed with ß actin antibody (1:1000; A2066, Sigma, Saint Louis, MO) or GAPDH antibody (gluceraldehyde-3-phosphate dehydrogenase; 1:500, SC-32233, Santa Cruz Biotechnology, Santa Cruz, CA), with donkey anti-rabbit-IRDye800CW (1:5000; #925-32213, Li-Cor) or goat anti-mouse- IRDye680LT (1:5000; #926-68020, Li-Cor) detection antibody, for use as a loading control. Western blots were repeated three times.

Hydroxyproline Assay. Quantification of hydroxyproline content in cell samples was performed using the method of Reddy and Enwemeka (Kesava Reddy and Enwemeka, 1996). For each specimen, cell samples were prepared in triplicate, with the addition of ascorbic acid, in order to quantify the amount of fibrillar collagen matrix present in the samples. The cells were seeded in cell culture plates (Corning®; 430167) at a density of 300,000 cells/plate and incubated overnight. After serum deprivation for 24 h, cells were then, in the presence of ascorbic acid (20µg/mL), treated with SubP (100 nM; Tocris, 1156) or TGFß-1(10 ng/mL; EMD Millipore, GF111) alone or in combination. A control without treatment was also included. After 72 h, all cell samples

were performed hydroxyproline analysis as follows (Blissett et al., 2009). The supernatant was removed and contents within the plates were centrifuged down and pipetted into individual 1.5mL Eppendorf tubes (Fisher Scientific, 05-408-129). All samples were then brought to a final volume of 200ul with a final concentration of 6N HCl and then hydrolyzed at 120°C for 3 h. 50µL of the supernatant was then transferred to a 96-well plate. All wells were evaporated to dryness in a 60°C oven. After which 100µL of chloromine T reagent was added to each sample and standard well, incubated at room temperature for 20 minutes. Thereafter, 100µL of Enrlich's reagent was added to each sample and standard well and incubated at 60°C for 90 minutes. Finally the absorbance of each sample was read at 560nm using a spectrophotometer. The amount of hydroxyproline in each sample was determined according to a standard curve of hydroxyproline, obtained using hydroxyproline from 0 to 1.0 µg/well. Hydroxyproline assay data (pg hydroxyproline) was normalized to µg of total protein, determined using a bicinchoninic acid protein assay kit (Thermo Scientific, Pierce, #23225). Collagen content was estimated by considering that hydroxyproline comprises 12.5% of collagen fibers (Kesava Reddy and Enwemeka, 1996).

TGFß-1 ELISA. Tenocytes or RDF cells were seeded in 96-well plates at a density of 10,000 cells/well overnight and then serum deprived for 24 h prior to treatment. Cells were treated with a standard concentration of 100, 10, 0.1, and 0 nM of SubP (1156, Tocris). After 24 h, supernatants of cultured cells were collected and used to measure total TGFß-1 concentrations using three distinct standard commercial ELISA kits (ADI-900-155, Enzo, sensitivity level of 3.3 pg/ml, Farmingdale, NY; BMS623, eBioscience, sensitivity level of 12 pg/ml, Austria; KAC1688, Invitrogen, sensitivity level of 15.6

pg/mL, Camarillo, CA) according to manufacturer's instructions. Each sample was run in triplicate.

Statistical Analyses

GraphPad PRISM v.6.02 was used for the statistical analyses. All data are expressed as mean ± SEM. P values of < 0.05 were considered significant for all comparisons. Proliferation and In-Cell western results were analyzed using two-way ANOVAs. One-way ANOVA or an un-paired *t*-test was used (two-tailed) to compare results of different treatments for other experiments. During the post-hoc ANOVA analyses for between group differences, the Turky correction method was used to reduce the chances of obtaining false-positive results (type I errors).

Results

Immunocytochemical staining: phenotype of cells

The vast majority of cultured tenocytes showed a clear elongated fibroblastic appearance under the microscope (Fig. 3-1 A-C), also evident by bright field visualization. All of the imaged tenocytes were immunopositive for the tenocyte markers tenomodulin and vimentin in the passages and serum concentrations used for experiments, thereby indicating a fibroblastic tenocytes phenotype. The expression of these markers in RDF cells was considered lower by immunocytochemistry (Fig. 3-1 D-F), but detectable versus the secondary only control (not shown).

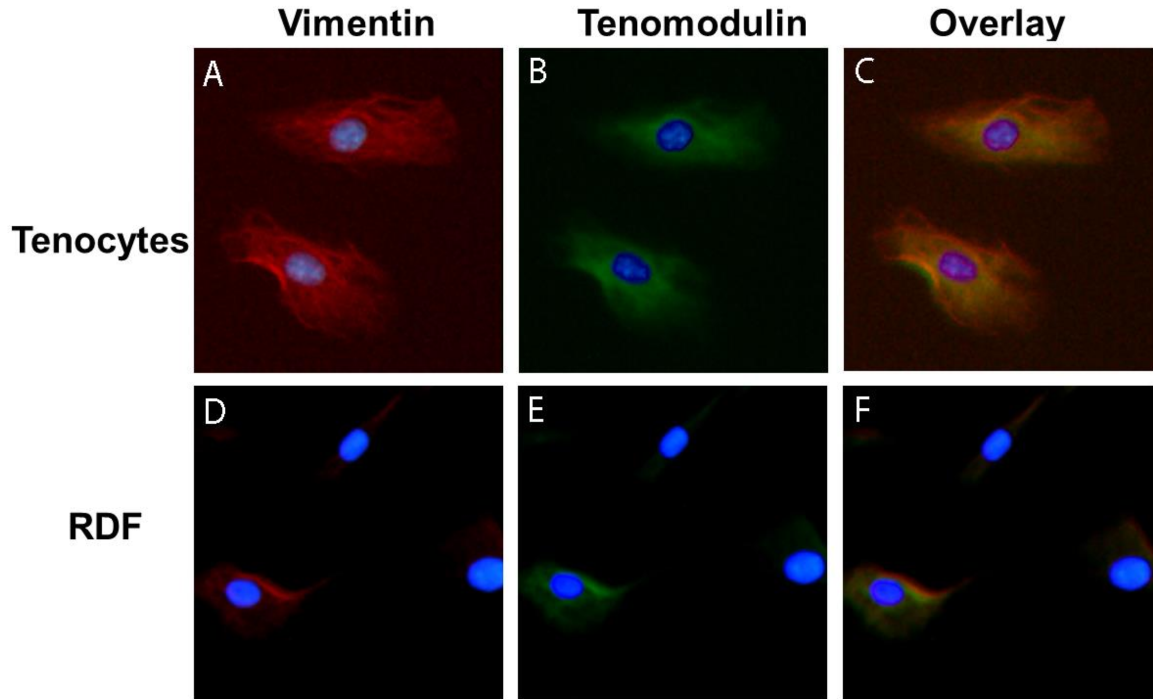

Figure 3.1 Immunocytochemical staining of primary cultures of rat flexor digitorum tendon cells and rat dermal fibroblasts (RDF). Nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI; blue). Most tendon cells are positive for vimentin as shown in (A) (red, tetramethylrhodamine isothiocyanate [TRITC]). (B) Cells showing immunoreaction for tenomodulin (green, fluorescein isothiocyanate [FITC]), a glycoprotein predominantly expressed in tendons and ligaments. RDF cells showed weaker but modest staining for both vimentin (D) and tenomodulin (E) under the same imaging conditions. An overlay of the fluorescence is shown in (C, F). Cells shown at 200 x magnification.

Effects of SubP and TGFß-1 Treatment on proliferation in primary tenocyte cultures

We evaluated proliferation on tenocytes incubated with either SubP or TGFß-1 at various concentrations for 72 hours, under serum-deprived conditions (1%). We observed a significant difference in the two-way ANOVA for different treatments ($p < 0.0001$). Post hoc analysis showed TGFß-1 treatment significantly stimulated tenocyte proliferation in a dose-dependent manner with the highest concentration (10ng/mL) inducing the most effect, compared to the untreated group, in three independent replications of these experiments, with duplicate wells per experiment (Fig. 3-2). This

effect was not enhanced by the addition of SubP. The effect of treatment of SubP alone on tenocyte proliferation was not significant. Similar results were achieved by BrdU Incorporation assay and AlamarBlue® Cell Viability assay (Fig. 3-3). Both the assays showed a greater increase with the combined treatment of both SubP (100nM) and TGFß-1 (10ng/ml); however, this experiment was repeated only once, with duplicate wells per treatment.

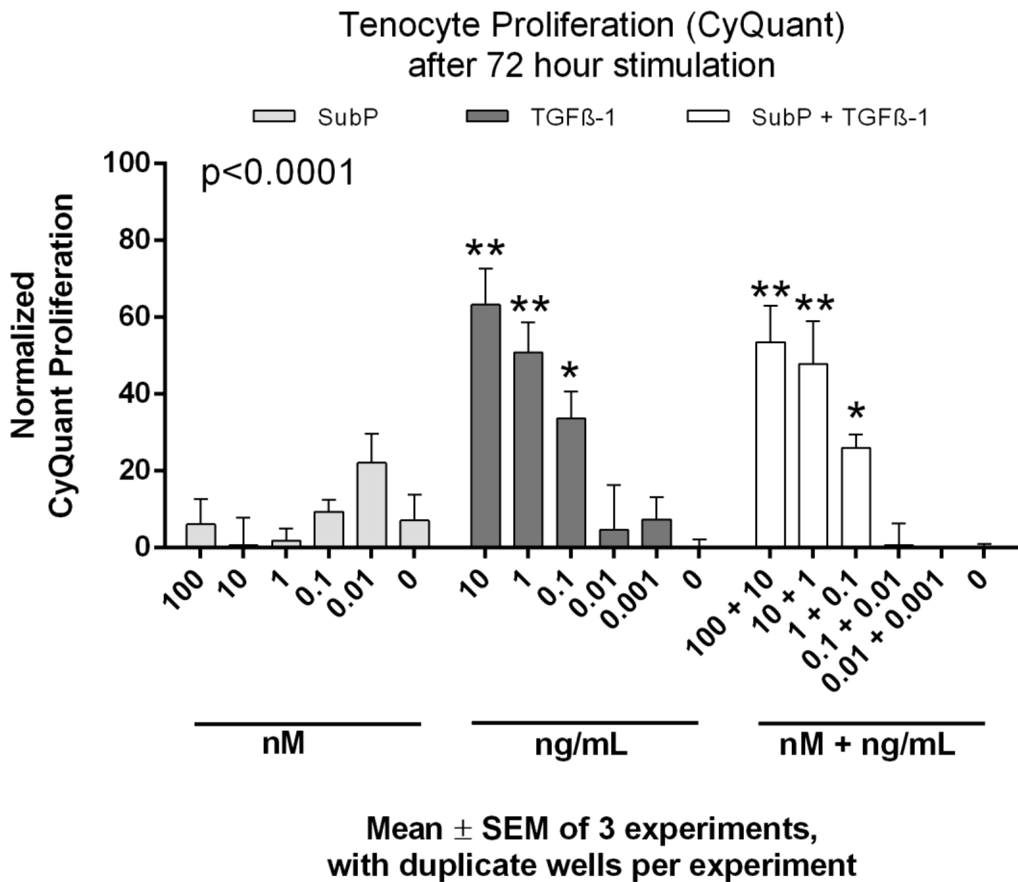

Fig. 3-2. Effects of SubP or TGFß-1 on tenocyte proliferation, normalized to FGF-2 levels. Tenocytes were cultured with SubP, TGFß-1 or their combination for 72 hours, under serum-deprived conditions (1%). The proliferation was evaluated by CyQUANT® NF Cell Proliferation Assay. Results shown are the mean of three experiments ± SEM, which were performed in duplicate for each experimental condition. *$p<0.05$ and **$p<0.01$ compared to untreated group (n=3), 2-way ANOVA with a Tukey post-test.

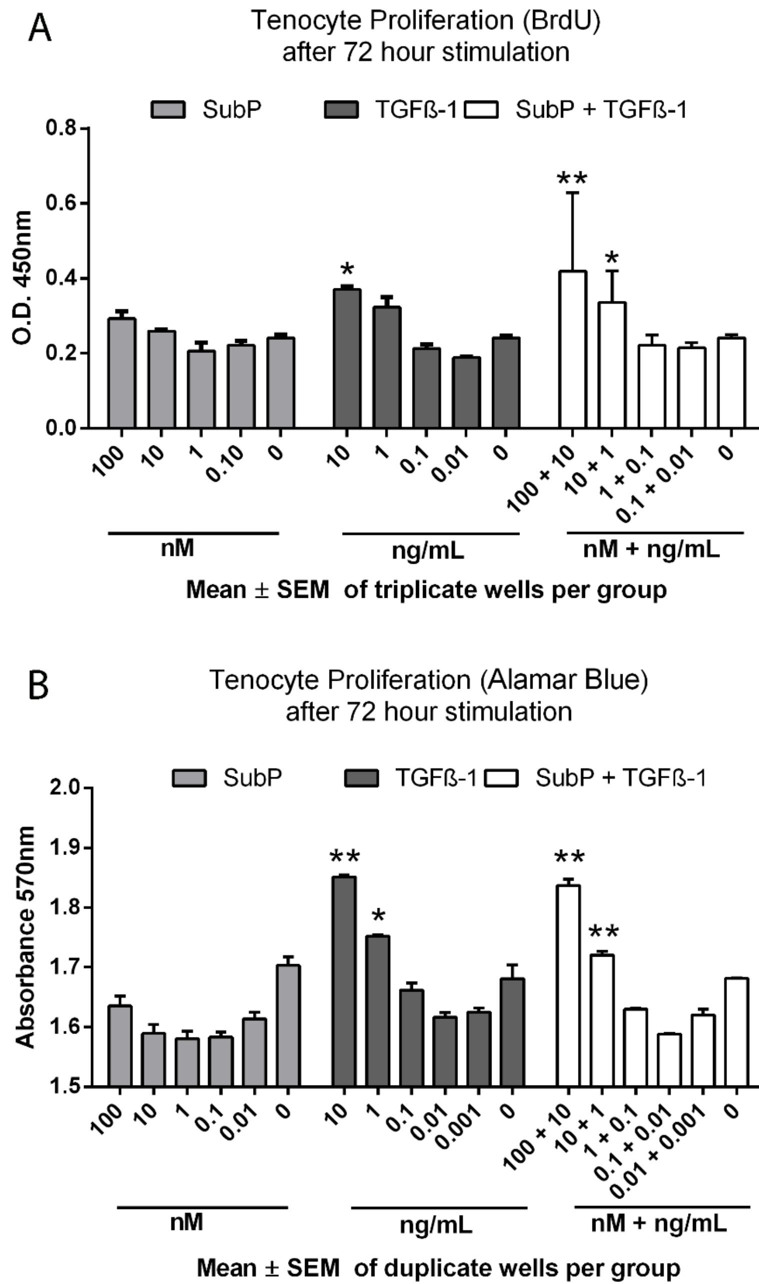

Fig. 3-3. Effects of SubP or TGFß-1 on tenocyte proliferation, under serum-deprived conditions (1%). Cell proliferation induced by SubP or TGFß-1 was confirmed by BrdU Incorporation assay (A) and AlamarBlue® Cell Viability assay (B). Results for each experiment condition shown are the mean ± SEM, which were performed in duplicate. *$p<0.05$ and **$p<0.01$ compared to untreated group, 2-way ANOVA with a Tukey post-test.

Effects of SubP and TGFß-1 Treatment on CCN2 expression in primary tenocytes and rat dermal fibroblasts cultures

We next examined CCN2 expression in tenocytes and RDF cells stimulated with either SubP, TGFß-1 or their combination at various concentrations for 48 hours using in-cell Western analysis. We observed a significant difference in the two-way ANOVA for different treatments (p= or p< 0.0001) in both tenocytes and RDF cells. Post hoc analysis showed CCN2 expression was significantly increased after 48 hours in tenocytes after incubation with SubP (100nM), TGFß-1 (10ng/mL) or the combination of SubP (100nM) plus TGFß-1 (10ng/mL). The combination treatment of SubP (25nM) and TGFß-1 (2.5 ng/ml) showed more CCN2 expression compared with SubP (25nM) or TGFß-1 (2.5 ng/ml) treatment alone (Fig. 3-4A).

CCN2 expression in RDF cells was significantly increased after incubation with TGFß-1 (10ng/ml) alone or SubP (100nM) plus TGFß-1 (10ng/ml) in combination for 48 hours (Fig. 3-4 B). SubP (100nM) alone trended higher than control wells, but did not reach statistical significance (p<0.0001). The combination treatment of SubP (25nM) and TGFß-1 (2.5 ng/ml) showed more CCN2 expression compared with SubP (25nM) treatment alone (Fig. 3-4B).

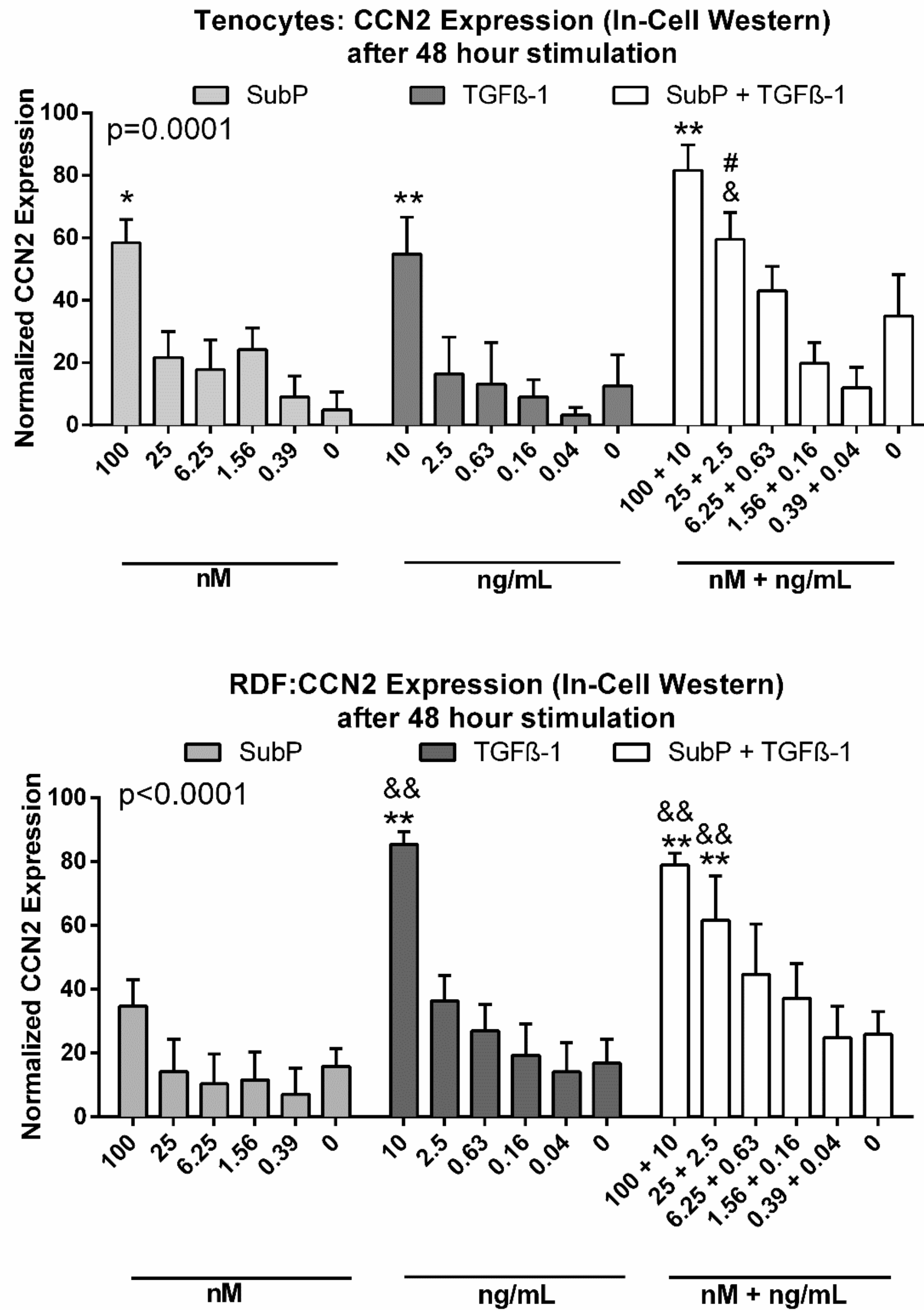

Fig. 3-4. Effects of SubP or TGFß-1 on CCN2 expression. Rat tenocytes (A) or RDF cells (B) were serum deprived (24 h) and treated with SubP, TGFß-1 or their combination at various concentrations to evaluate a dose response, under continued serum deprivation condition (1%). CCN2 expression was assessed by in-cell Western analysis at 48 hours after incubation. Results shown are the mean of 3 experiments ± SEM, which were performed in duplicate for each experimental condition. **p< 0.01 compared to untreated group. [&&] p<0.01 compared to SubP treatment, 2-way ANOVA with a Tukey post-test.

In the next series of experiments, we confirmed the ability of SubP (100nM) and TGFß-1 (10ng/ml) to up-regulate CCN2 expression in primary tenocytes and RDF cells cultures using Western blot method. Western blot analysis was followed by densitometry using Image Studio (LI-COR Biosciences, Lincoln, NE). We observed a significant difference in the one-way ANOVA for different treatments in both tenocytes and RDF cells ($p = 0.004$ and $p = 0.04$). Post hoc analysis revealed that treatment of tenocytes with SubP (100nM) or TGFß-1 (10ng/ml) alone induced increases in CCN2 protein expression levels in tenocytes, compared to control (untreated) cultures (Fig. 3-5. A, B). No significant differences were seen with the combination treatment, compared to TGFß-1 treatments alone. The results of RDF cells differed. Both TGFß-1 (10 ng/ml) treatment alone and the combination treatment of SubP (100nM) and TGFß-1 (10 ng/ml) induced increased CCN2 protein expression, compared to control (untreated culture). However, SubP (100nM) treatment alone had no effect on CCN2 expression (Fig. 3-5. C, D).

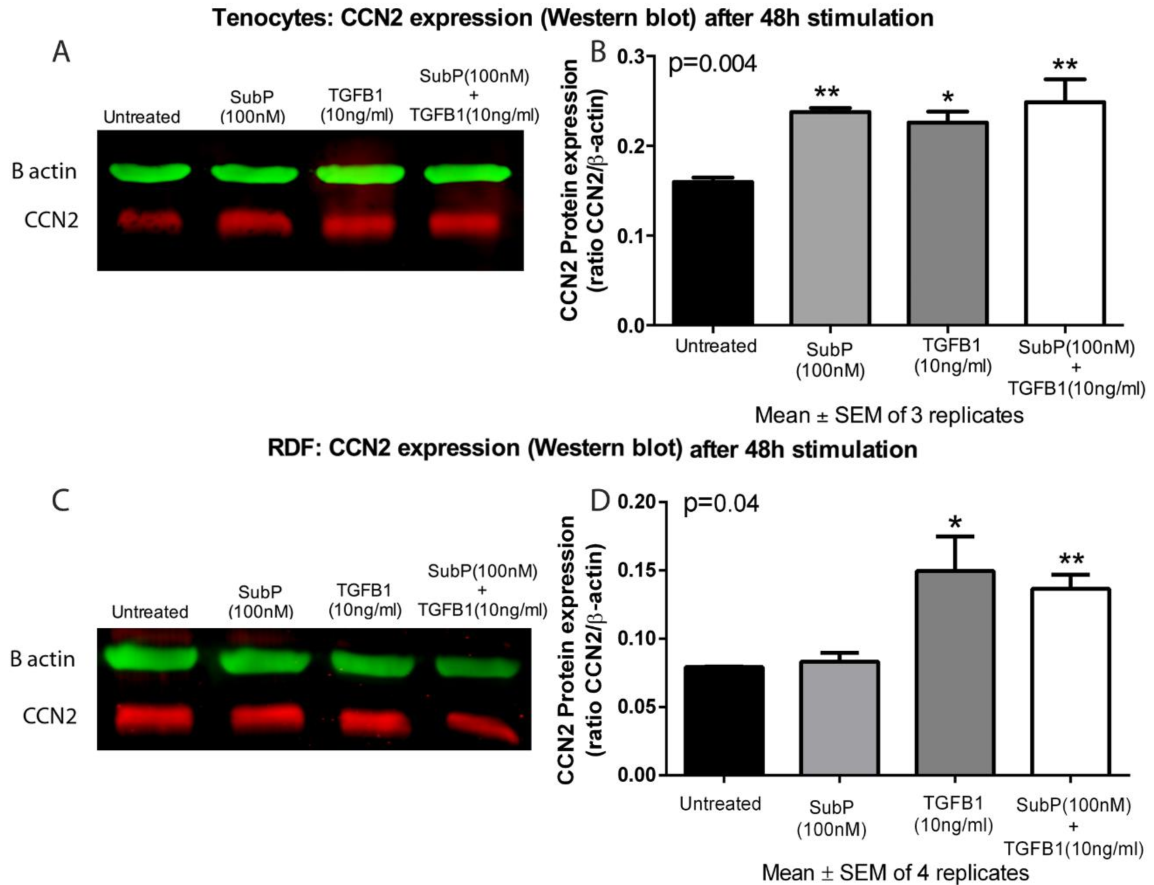

Fig. 3-5. Effects of SubP or TGFß-1 on CCN2 expression in serum-deprived conditions (1%). (A) A representative Western blot of lysates collected from tenocytes stimulated with SubP (100nM), TGFß-1 (10 ng/ml) or their combination for 48 h, probed with anti-CCN2 (at 37 kDa). ß actin was used as a loading control (at 42 kDa). (B) Densitometric analysis of three replicate Western Blots, showing ratio of CCN2 bands normalized with ß actin levels. (C) A representative Western blot of RDF cells stimulated with SubP (100nM) or TGFß-1 (10 ng/ml) or combination for 48 h, probed with anti-CCN2 (at 37 kDa) and anti-ß actin (at 42 kDa). (D) Densitometric analysis of four replicate Western blots, showing ratio of CCN2 bands normalized to ß actin protein. The results are presented as the mean density of 3 or 4 experiments ± SEM. *p< 0.05, **p< 0.01 compared to untreated group, 1-way ANOVA with a Tukey post-test.

The ability of SubP and TGFß-1 to up-regulate CCN2 expression in primary tenocytes cultures or RDF cells was also confirmed using immunohistochemical staining of cells grown in chamber slides, under serum deprived (1%) conditions beginning 24 hours prior to the onset of treatments. We observed a significant difference in the one-way ANOVA for different treatments in both tenocytes and RDF cells ($p < 0.0001$). For tenocytes, the untreated group showed low CCN2 immunostaining in the cells (Fig. 3-6 A). Post hoc analysis for image analysis quantification confirmed CCN2 immunostaining in tenocytes was increased with either SubP (100nM) or TGFß-1 (10ng/ml) alone or the combination treatment (Fig. 3-6 A, B). The effect of TGFß-1 (10ng/ml) alone on CCN2 expression was greater than SubP (100nM) alone, and this effect of TGFß-1 was significantly improved by addition of SubP (100nM) (Fig. 3-6 A, B).

RDF cells showed similar results (Fig. 3-6 C, D). CCN2 immunostaining was not evident in untreated group (Fig. 3-6 C), yet was increased in RDF cells treated with TGFß-1 (10ng/ml) or SubP (100nM) plus TGFß-1 (10ng/ml) in combination for 48 hours (Fig. 3-6 C). Image analysis quantification confirmed this increase, compared to the untreated RDF cells (Fig. 3-6 D). Furthermore, the effect of combination treatment on CCN2 expression per cell was greater than the treatment of SubP (100nM) alone (Fig. 3-6 D).

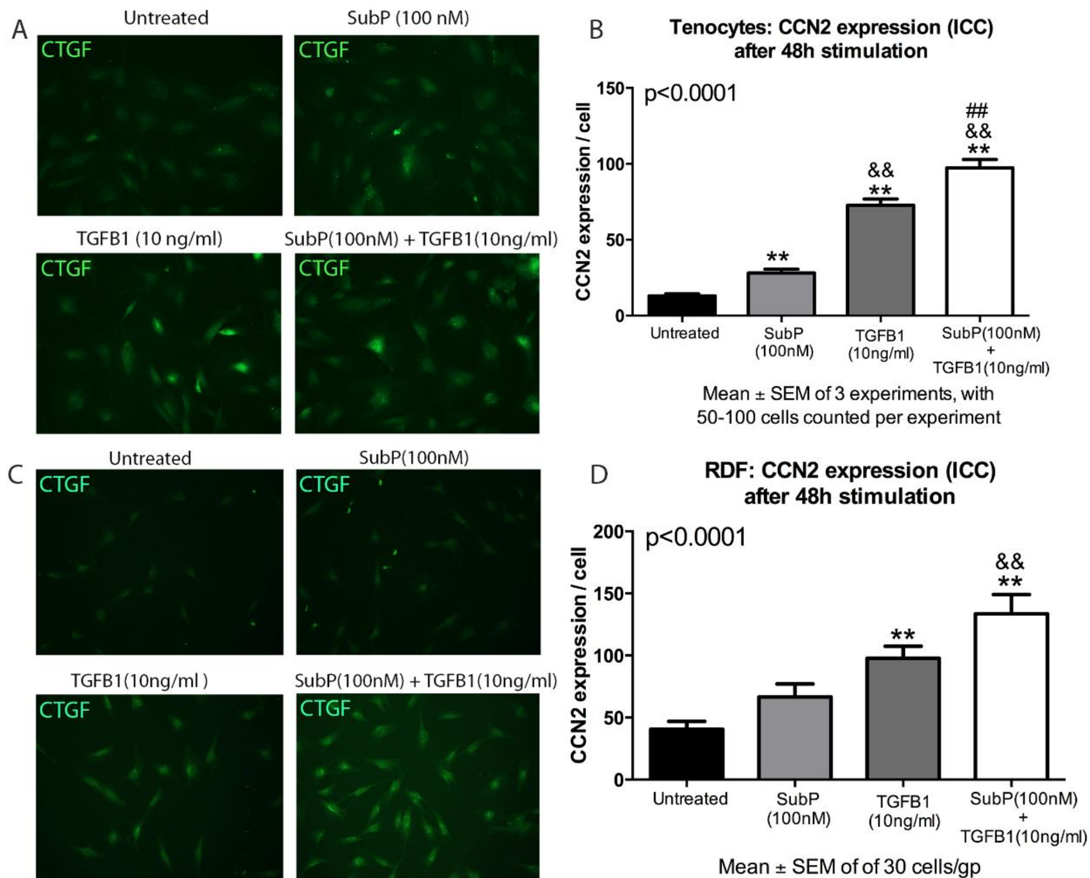

Fig. 3-6. Effects of SubP or TGFß-1 on CCN2 expression in serum-deprived conditions (1%). (A, C) Representative CCN2 immunostaining in primary tenocytes (A) or RDF cells (C) treated with SubP (100nM) or TGFß-1 (10 ng/ml) or combination for 48 h. (B, D) Quantification of CCN2 immunostaining per cell in tenocytes (B) or RDF cells (D). The results are presented as the mean density of three independent experiments ± SEM. $^{**}p<0.01$ compared to untreated group. $^{\&\&}p<0.01$ compared to SubP (100nM) treatment. $^{\#\#}p<0.01$ compared to TGFß-1 (10ng/ml) treatment, 1-way ANOVA with a Tukey post-test.

The Effect of SubP and TGFβ-1 on collagen production in primary tenocytes cultures

To determine if SubP has an effect on collagen production in our primary tenocytes culture system, we used hydroxyproline assay to measure the amount of collagen deposition in cell samples. Tenocytes were treated in the presence of ascorbic acid (20 μg/mL). The amount of soluble collagen present in the conditioned media was not assessed. BCA assay was used to normalize the total protein levels due to potential

variation in proliferation rates. We observed a significant difference in the one-way ANOVA for different treatments in tenocytes ($p < 0.0001$). As shown in Fig. 3-7, post hoc analysis showed collagen was significantly increased with TGFß-1 and the combination treatment, compared to untreated control cells, and compared to SubP treatment alone. Little or no variation occurred in the hydroxyproline content for samples treated with SubP alone.

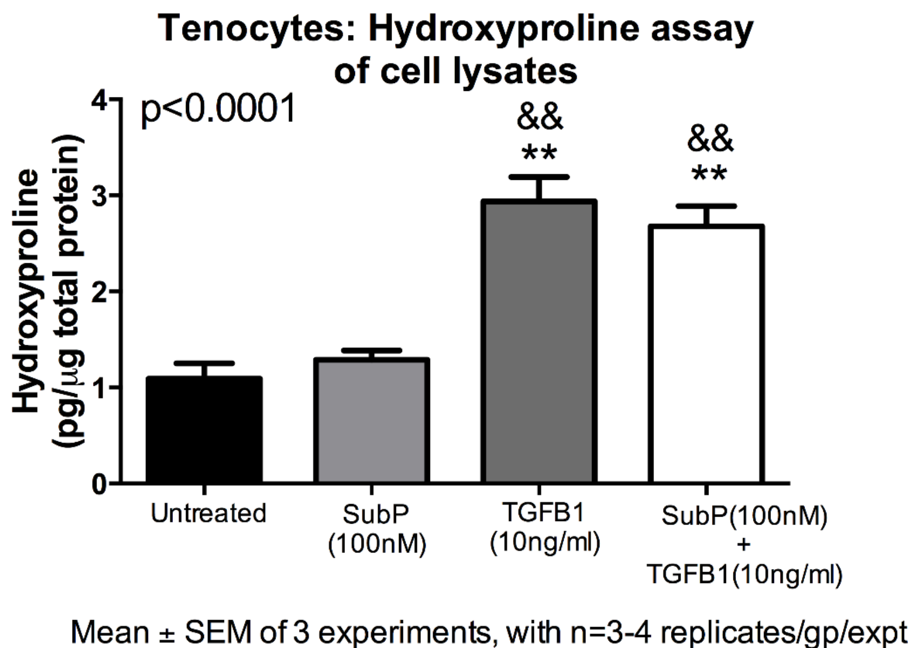

Fig. 3-7. Effects of SubP or TGFß-1 on collagen deposition in tenocytes. Primary tenocytes were serum deprived (24 h) and treated with SubP (100nM) or TGFß-1 (10 ng/ml) or combination for 72 h, with continued serum-deprived conditions (1%). Collagen deposition was assessed by hydroxyproline assay. Bar graphs represent data from measured values. The results are presented as the mean density of 3 experiments ± SEM. $^{**}p<0.01$ compared to untreated group. $^{\&\&}p<0.01$ compared to SubP (100nM) treatment, 1-way ANOVA with a Tukey post-test.

The Effect of SubP onTGFß-1 production in primary tenocytes and rat dermal fibroblasts cultures

In our study, three different ELISA kits with three different levels of sensitivity, showed that TGFß-1 levels in the supernatant after SubP stimulation in both tenocytes and RDF cells were at or below the level of detection and were therefore not significantly up-regulated above untreated levels. We also examined TGFß-1 expression in lysates collected from tenocytes stimulated with SubP using western blot analysis. Similarly, no significant differences were detected post-treatment, compared to the control (untreated) group (data not shown). These results indicate that SubP acts independently of TGFß-1 in its induction of CCN2 production.

Discussion

This study establishes primary rat flexor digitorum tendon cells as a valid model for studying the effects of the fibrosis-related mediators SubP and TGFß-1. Other groups have successfully grown primary human or rat Achilles tendon cells in culture (Luo et al., 2009; Backman et al., 2011b). However, this is the first attempt, to our knowledge, to isolate tenocytes from the flexor digitorum tendon to determine the effects of fibrotic mediators. The majority of cultured cells showed characteristics of a fibroblastic elongated phenotype and expressed vimentin and tenomodulin (tenocyte markers), which were retained after multiple passages, confirming that our cultures consisted almost entirely of tenocytes, consistent with Backman et al (Backman et al., 2011b). However, it should be noted that other cell types could also be present, such as chondrocytes, endothelial cells (Kleiner, 1998), and nerve cells (Danielson et al., 2006).

This study found that isolated flexor digitorum tenocytes respond to both SubP and TGFß-1, similar to studies of isolated Achilles tendon cells from rats and humans (Luo et al., 2009; Backman et al., 2011a; Fong et al., 2013). However, this is the first report, to our knowledge, to demonstrate that SubP treatment of primary tenocytes directly induces CCN2 production. Interestingly, the responsiveness of RDF cells differed from tenocytes, with little or no induction of CCN2 production after SubP treatment. The neuropeptide SubP increases in several inflammatory conditions, including painful tendinopathies, rotator cuff tears, and arthritis (reviewed in (O'Connor et al., 2004; Riley, 2008; Steinhoff et al., 2014)). SubP participates in many inflammatory and fibrotic processes and is produced not only in neurons, but also by

tenocytes (Backman et al., 2011b) and immune cells such as mast cells (Toyoda et al., 2000; Rani et al., 2009), macrophages (Fedorczyk et al., 2010; Germonpre et al., 1999; Clark et al., 2004), dendritic cells (Lambrecht et al., 1999), and T lymphocytes (Lambrecht et al., 1999). The release of SubP is believed to have both autocrine and paracrine effects, including cytokine release from multiple tissue types. Our findings indicate that the mechanism of increased CCN2 production by SubP was distinct from TGFß-1, since SubP treatment did not increase TGFß-1 production.

Backman et al (2011) indicated that SubP, produced by human tenocytes in response to mechanical loading, may regulate tenocyte proliferation through an autocrine loop involving its receptor, neurokinin 1 receptor (NK-1R) (Backman et al., 2011b). We did not mechanically load the cells in our system and do not know if this autocrine production exists in our model. However, we do know that recombinant SubP treatment had no effect on collagen production or cell proliferation in our rat flexor digitorum tenocytes, although it did elicit an increase in CCN2. This finding combined with those of Backman's may indicate that additional stress (e.g. mechanical loading) or cofactors were needed to induce fibroblast proliferation and matrix changes in our system. There are conflicting reports on the effects of SubP on proliferation and ECM production in other cell types as well. Dehlin et al reported that rat cardiac fibroblasts stimulated with SubP showed no increase in collagen I synthesis (Dehlin et al., 2013). Kumaran et al demonstrated that SubP increases proliferation in isolated cardiac fibroblasts, but did not increase collagen secretion (Kumaran and Shivakumar, 2002). *In vitro* experiments with colonic fibroblasts show that SubP, in the presence of TGFß-1 and IGF-1, stimulates collagen synthesis, while SubP alone does not (Koon et al., 2010). Further, it should be

noted that Andersson et al have reported exogenously administered SubP accelerated hypercellularity in tendon tissue and enhanced paratendinitis in response to Achilles tendon overuse in a rat tendinopathy model (Andersson et al., 2011). The multicellular environment present in this *in vivo* study was not present in our monoculture system.

As mentioned, we observed an increase in TGFß-1-induced cell proliferation and extracellular matrix production (both CCN2 and collagen) in tenocytes. TGFß-1 and CCN2 have both been used as serum biomarkers of fibrogenic disease and recent information highlights the role of TGFß-1 and CCN2 in tissue repair responses that lead to matrix deposition and tissue remodeling (Igarashi et al., 1993; Abdelmagid et al., 2012; Gao et al., 2013; Abrahams et al., 2014; Fisher et al., 2015). CCN2 is generally considered a downstream mediator of TGFß-1 and is produced by multiple cell types (Grotendorst, 1997; Song et al., 2007). Studies in the murine mesenchymal stem cell line showed that TGFß-1-induced CCN2 expression leads to fibroblast proliferation and extracellular matrix deposition (Song et al., 2007). Overproduction of these proteins has been linked to excessive scar tissue deposition in muscle and tendon in many chronic inflammation diseases (Leask and Abraham, 2004; Li et al., 2006; Smith et al., 2007; Lipson et al., 2012). Our results in tenocytes confirm the importance of TGFß-1 in driving cell proliferation and extracellular matrix production.

After confirming the effects of SubP and TGFß-1 on extracellular matrix production and cell proliferation by tenocytes, we attempted to determine if the effects seen with SubP were caused by SubP inducement of TGFß-1 production in tenocytes. Although SubP is able to modulate cytokine production in various conditions, there are only few reports examining the effect of SubP on TGFß-1 production. Yaraee et al (2009)

reported that SubP can directly modulate the release of TGFß-1 from a human bronchial epithelial cell line (Yaraee and Ghazanfari, 2009). Hu D et al (2002) found that dermal fibroblasts derived from human normal skin cultured with 25 ng/mL of SubP had more TGFß-1 mRNA expression compared to the control group (Hu et al., 2002). Koon et al (2010) showed that SubP plays a significant role in the pathogenesis of intestinal fibrosis via its stimulatory effect on stimulating TGFß-1 production. However, in our study, there were no detectable supernatant TGFß-1 in either primary cell type using ELISA and western blot. Species differences in cytokine response profiles and the detection system used may account for these varying results (Barbe et al., 2008). Although we fairly ruled out detection system differences by our use of three different ELISA kits with different sensitivities (with ranges from 3.3 pg/ml to 15.6 pg/ml), it is possible that the levels of TGFß-1 produced by these cell types were too low to be detected. That said, it is also possible that the mechanism of CCN2 production induced by SubP is independent of TGFß-1. If SubP was inducing sufficient levels of TGFß-1, an effect on proliferation would be expected.

Furthermore, in this study, the tenocytes were collected from forelimb rather than hindlimb tendons, differing from Backman's human Achilles tenocytes culture (Backman et al., 2011b), and importantly, the tenocytes used in this study were never mechanically loaded. Many studies show that mechanical loading, stretch or injury is required to induce matrix changes in fibroblasts/tenocytes. For example, Archambault et al reported that stretch and exposure to inflammatory cytokines are needed (one or more) to induce changes in matrix proteins expression in tenocytes, and that combination was synergistic (Archambault et al., 2002). This finding suggests that the potentially matrix damaging

effect of combining mechanical loading with pre-existing inflammatory conditions may have significant implications in fibrosis. However, neither was present in our fibroblasts/tenocytes culture system, which may explain why there was no increase of TGFß-1 or collagen production in our cells after SubP stimulation. In the future, examine TGFß-1 or collagen production again on tenocytes collected from flexor digitorum tendons of 18-week HRHF rats (young adult female rats that are performed with high repetition high force hand pulling tasks for 18 weeks) after SubP stimulation could help to address this question.

In summary, we demonstrated that both substance P and TGFß-1 can induce CCN2 production in rat tenocytes. We further demonstrated that SubP and TGFß-1 did not act in a consistently additive or synergistic fashion to promote fibroblast proliferation and collagen production. Lastly, this effect may be TGFß-1 independent since no TGFß-1 protein was detected after SubP treatment. Unlike TGFß-1, SubP did not affect proliferation of tenocytes or induce collagen production on its own. These data suggest that both SubP and TGFß-1 have distinct fibrogenic actions on tenocytes and both may be involved in tendinosis observed in the animal models and patients.

CHAPTER 4

SUMMARY

The primary purpose of this study was to examine the role of anti-fibrogenic protein, IFNγ and fibrogenic mediators, TGFß-1, CCN2 and SubP in fibrosis occurring in an *in vivo* rat model of upper extremity WMSD and in fibroblasts *in vitro*.

We used histological and immunochemical techniques to access fibrogenic and anti-fibrogenic protein production *in vivo*. The immunohistochemical quantification results show increases in TGFß-1, IFNγ and CCN2 in tissues with long-term (18-week) HRHF task. TGFß-1 and IFNγ immunoexpression was increased in small cells on the perimeter of myofibers and in flexor digitorum tenocytes of 18-week HRHF rats, compared to FRC rats. CCN2 immunostaining was also enhanced in 18-week HRHF rat muscles in small cells on the perimeter of myofibers and endomysium, compared to the FRC rats. This confirms the hypothesis that the inflammatory cytokine responses resolve early, and that fibrotic tissue responses (specifically TGFß-1 and CCN2) will increase significantly in flexor digitorum muscles with prolonged performance of a high demand repetitive task for 18 weeks, matching tissue findings in tissue biopsies collected from patients with chronic WMSDs.

We further used cell-based assays to address the interactions among fibrosis-related mediators (specifically TGFß-1, CCN2 and SubP) *in vitro*. We identified that SubP can up-regulate protein expression levels of CCN2 in both rat primary tenocyes and rat dermal fibroblasts cell line (i.e., RDF cells). However, we did not observe that SubP increases TGFß-1 production directly in either primary cell type. We further

demonstrated that SubP and TGFß-1 did not act in a consistently addictive or synergistic fashion to promote fibroblast proliferation and collagen production. Unlike TGFß-1, SubP was not able to affect tenocytes proliferation and collagen production on its own. These data indicate that both SubP and TGFß-1 have distinct fibrotic actions on tenocytes and both may be involved in tendinosis observed in the animal models and patients. Our data supports the hypothesis that SubP acts directly through CCN2 to alter primary tenocytes proliferation and extracellular matrix production *in vitro*, independently of TGFß-1 (Fig. 4-1).

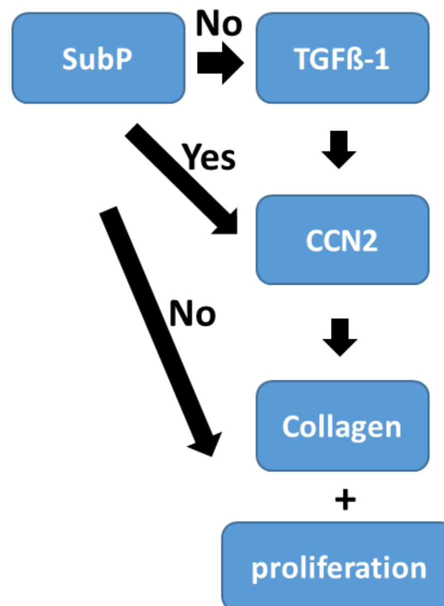

Figure 4-1. Schematic diagram showing pathways hypothesized to lead to fibroblast proliferation and collagen production *in vitro.* Our data supports the hypothesis that SubP acts directly through CCN2 to alter primary tenocytes proliferation and extracellular matrix production *in vitro*, independently of TGFß-1.

REFERENCES

Abdelmagid SM, Barr AE, Rico M, Amin M, Litvin J, Popoff SN, Safadi FF, Barbe MF. 2012. Performance of repetitive tasks induces decreased grip strength and increased fibrogenic proteins in skeletal muscle: role of force and inflammation. PloS one 7:e38359.

Abrahams AC, Habib SM, Dendooven A, Riser BL, van der Veer JW, Toorop RJ, Betjes MGH, Verhaar MC, Watson CJE, Nguyen TQ, Boer WH. 2014. Patients with Encapsulating Peritoneal Sclerosis Have Increased Peritoneal Expression of Connective Tissue Growth Factor (CCN2), Transforming Growth Factor-β1, and Vascular Endothelial Growth Factor. PloS one 9:e112050.

Al-Shatti T, Barr AE, Safadi FF, Amin M, Barbe MF. 2005. Increase in inflammatory cytokines in median nerves in a rat model of repetitive motion injury. J Neuroimmunol 167:13-22.

Alberts B JA, Lewis J, et al. 2002. Molecular Biology of the Cell. 4th edition. In: Fibroblasts and Their Transformations: The Connective-Tissue Cell Family. New York: Garland Science.

Andersson G, Backman LJ, Scott A, Lorentzon R, Forsgren S, Danielson P. 2011. Substance P accelerates hypercellularity and angiogenesis in tendon tissue and enhances paratendinitis in response to Achilles tendon overuse in a tendinopathy model. British journal of sports medicine 45:1017-1022.

Archambault J, Tsuzaki M, Herzog W, Banes AJ. 2002. Stretch and interleukin-1β induce matrix metalloproteinases in rabbit tendon cells in vitro. Journal of Orthopaedic Research 20:36-39.

Arnott JA, Nuglozeh E, Rico MC, Arango-Hisijara I, Odgren PR, Safadi FF, Popoff SN. 2007. Connective tissue growth factor (CTGF/CCN2) is a downstream mediator for TGF-beta1-induced extracellular matrix production in osteoblasts. J Cell Physiol 210:843-852.

Arnott JA, Zhang X, Sanjay A, Owen TA, Smock SL, Rehman S, DeLong WG, Safadi FF, Popoff SN. 2008. Molecular Requirements for Induction of CTGF Expression by TGF-β1 in Primary Osteoblasts. Bone 42:871-885.

Aslan H, Kimelman-Bleich N, Pelled G, Gazit D. 2008. Molecular targets for tendon neoformation. The Journal of clinical investigation 118:439-444.

Backman LJ, Andersson G, Wennstig G, Forsgren S, Danielson P. 2011a. Endogenous substance P production in the Achilles tendon increases with loading in an in vivo model of tendinopathy-peptidergic elevation preceding tendinosis-like tissue changes. Journal of musculoskeletal & neuronal interactions 11:133-140.

Backman LJ, Fong G, Andersson G, Scott A, Danielson P. 2011b. Substance P Is a Mechanoresponsive, Autocrine Regulator of Human Tenocyte Proliferation. PLoS ONE 6:e27209.

Balayssac D, Ling B, Ferrier J, Pereira B, Eschalier A, Authier N. 2014. Assessment of thermal sensitivity in rats using the thermal place preference test: description and application in the study of oxaliplatin-induced acute thermal hypersensitivity and inflammatory pain models. Behavioural pharmacology 25:99-111.

Bancroft GJ, Schreiber RD, Unanue ER. 1991. Natural immunity: AT-cell-independent pathway of macrophage activation, defined in the scid mouse. Immunological reviews 124:5-24.

Barbe MF, Barr AE. 2006. Inflammation and the pathophysiology of work-related musculoskeletal disorders. Brain, Behavior, and Immunity 20:423-429.

Barbe MF, Elliott MB, Abdelmagid SM, Amin M, Popoff SN, Safadi FF, Barr AE. 2008. Serum and tissue cytokines and chemokines increase with repetitive upper extremity tasks. Journal of Orthopaedic Research 26:1320-1326.

Barbe MF, Gallagher S, Massicotte VS, Tytell M, Popoff SN, Barr-Gillespie AE. 2013a. The interaction of force and repetition on musculoskeletal and neural tissue responses and sensorimotor behavior in a rat model of work-related musculoskeletal disorders. BMC Musculoskelet Disord 14:303.

Barbe MF, Gallagher S, Popoff SN. 2013b. Serum Biomarkers as Predictors of Stage of Work-related Musculoskeletal Disorders. Journal of the American Academy of Orthopaedic Surgeons 21:644-646.

Baroni GS, D'Ambrosio L, Curto P, Casini A, Mancini R, Jezequel AM, Benedetti A. 1996. Interferon gamma decreases hepatic stellate cell activation and extracellular matrix deposition in rat liver fibrosis. Hepatology 23:1189-1199.

Baumert J-T, Sparmann G, Emmrich J, Liebe S, Jaster R. 2006. Inhibitory effects of interferons on pancreatic stellate cell activation. World Journal of Gastroenterology : WJG 12:896-901.

Bedair HS, Karthikeyan T, Quintero A, Li Y, Huard J. 2008. Angiotensin II Receptor Blockade Administered After Injury Improves Muscle Regeneration and Decreases Fibrosis in Normal Skeletal Muscle. The American journal of sports medicine 36:1548-1554.

Beinborn M, Blum A, Hang L, Setiawan T, Schroeder JC, Stoyanoff K, Leung J, Weinstock JV. 2010. TGF-beta regulates T-cell neurokinin-1 receptor internalization and function. Proceedings of the National Academy of Sciences of the United States of America 107:4293-4298.

Blissett AR, Garbellini D, Calomeni EP, Mihai C, Elton TS, Agarwal G. 2009. Regulation of Collagen Fibrillogenesis by Cell-surface Expression of Kinase Dead DDR2. Journal of Molecular Biology 385:902-911.

Blobe GC, Schiemann WP, Lodish HF. 2000. Role of Transforming Growth Factor β in Human Disease. New England Journal of Medicine 342:1350-1358.

BLS. 2013. Nonfatal occupational injuries and illnesses requiring days away from work, 2012. www.bls.gov/news.release/pdf/osh2.pdf. USDL-13-2257, November 26, 2013 Washington, DC.

Burssens P, Steyaert A, Forsyth R, van Ovost EJ, De Paepe Y, Verdonk R. 2005. Exogenously administered substance P and neutral endopeptidase inhibitors stimulate fibroblast proliferation, angiogenesis and collagen organization during Achilles tendon healing. Foot & ankle international 26:832-839.

Carlsson O, Schizas N, Li J, Ackermann PW. 2011. Substance P injections enhance tissue proliferation and regulate sensory nerve ingrowth in rat tendon repair. Scandinavian Journal of Medicine & Science in Sports 21:562-569.

Carp SJ, Barbe MF, Winter KA, Amin M, Barr AE. 2007. Inflammatory biomarkers increase with severity of upper-extremity overuse disorders. Clin Sci (Lond) 112:305-314.

Chen C-C, Lau LF. 2009. Functions and mechanisms of action of CCN matricellular proteins. The International Journal of Biochemistry & Cell Biology 41:771-783.

Chen MH, Huang YC, Sun JS, Chao YH, Chen MH. 2015. Second messengers mediating the proliferation and collagen synthesis of tenocytes induced by low-level laser irradiation. Lasers in medical science 30:263-272.

Chen Y, Chen J, Dong J, Liu W. 2005. Antifibrotic effect of interferon gamma in silicosis model of rat. Toxicology letters 155:353-360.

Chéret J, Lebonvallet N, Buhé V, Carre JL, Misery L, Le Gall-Ianotto C. 2014. Influence of sensory neuropeptides on human cutaneous wound healing process. Journal of dermatological science 74:193-203.

Chikenji T, Gingery A, Zhao C, Passe SM, Ozasa Y, Larson D, An KN, Amadio PC. 2014. Transforming growth factor-beta (TGF-beta) expression is increased in the subsynovial connective tissues of patients with idiopathic carpal tunnel syndrome. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 32:116-122.

Chuen FS, Chuk CY, Ping WY, Nar WW, Kim HL, Ming CK. 2004. Immunohistochemical Characterization of Cells in Adult Human Patellar Tendons. Journal of Histochemistry & Cytochemistry 52:1151-1157.

Cicha I, Goppelt-Struebe M. 2009. Connective tissue growth factor: Context-dependent functions and mechanisms of regulation. Biofactors 35:200-208.

Clark BD, Al-Shatti TA, Barr AE, Amin M, Barbe MF. 2004. Performance of a high-repetition, high-force task induces carpal tunnel syndrome in rats. J Orthop Sports Phys Ther 34:244-253.

Cohn RD, van Erp C, Habashi JP, Soleimani AA, Klein EC, Lisi MT, Gamradt M, ap Rhys CM, Holm TM, Loeys BL, Ramirez F, Judge DP, Ward CW, Dietz HC. 2007. Angiotensin II type 1 receptor blockade attenuates TGF-[beta]-induced failure of muscle regeneration in multiple myopathic states. Nat Med 13:204-210.

Danielson P, Alfredson H, Forsgren S. 2006. Immunohistochemical and histochemical findings favoring the occurrence of autocrine/paracrine as well as nerve-related cholinergic effects in chronic painful patellar tendon tendinosis. Microscopy Research and Technique 69:808-819.

De Winter P, Leoni P, Abraham D. 2008. Connective tissue growth factor: Structure–function relationships of a mosaic, multifunctional protein. Growth factors 26:80-91.

Dehlin HM, Manteufel EJ, Monroe AL, Reimer MH, Jr., Levick SP. 2013. Substance P acting via the neurokinin-1 receptor regulates adverse myocardial remodeling in a rat model of hypertension. International journal of cardiology 168:4643-4651.

Derby BM, Reichensperger J, Chambers C, Bueno RA, Suchy H, Neumeister MW. 2012. Early growth response factor-1: expression in a rabbit flexor tendon scar model. Plastic and reconstructive surgery 129:435e-442e.

Diaz KT, Skaria S, Harris K, Solomita M, Lau S, Bauer K, Smaldone GC, Condos R. 2012. Delivery and safety of inhaled interferon-gamma in idiopathic pulmonary fibrosis. Journal of aerosol medicine and pulmonary drug delivery 25:79-87.

Diegelmann RF, Evans MC. 2004. Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci 9:283-289.

Elliott MB, Barr AE, Clark BD, Amin M, Amin S, Barbe MF. 2009. High force reaching task induces widespread inflammation, increased spinal cord neurochemicals and neuropathic pain. Neuroscience 158:922-931.

Elliott MB, Barr AE, Clark BD, Wade CK, Barbe MF. 2010. Performance of a repetitive task by aged rats leads to median neuropathy and spinal cord inflammation with associated sensorimotor declines. Neuroscience 170:929-941.

Fan TP, Hu DE, Guard S, Gresham GA, Watling KJ. 1993. Stimulation of angiogenesis by substance P and interleukin-1 in the rat and its inhibition by NK1 or interleukin-1 receptor antagonists. British Journal of Pharmacology 110:43-49.

Fedorczyk JM, Barr AE, Rani S, Gao HG, Amin M, Amin S, Litvin J, Barbe MF. 2010. Exposure-dependent increases in IL-1beta, substance P, CTGF, and tendinosis in flexor digitorum tendons with upper extremity repetitive strain injury. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 28:298-307.

Felipe CD, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJH, Laird JMA, Belmonte C, Cervero F, Hunt SP. 1998. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature 392:394-397.

Fine A, Goldstein RH. 1987. The effect of transforming growth factor-beta on cell proliferation and collagen formation by lung fibroblasts. Journal of Biological Chemistry 262:3897-3902.

Fisher P, Zhao Y, Rico M, Massicotte V, Wade C, Litvin J, Bove G, Popoff S, Barbe M. 2015. Increased CCN2, substance P and tissue fibrosis are associated with sensorimotor declines in a rat model of repetitive overuse injury. J Cell Commun Signal:1-18.

Fitzner B, Brock P, Nechutova H, Glass Ä, Karopka T, Koczan D, Thiesen H-J, Sparmann G, Emmrich J, Liebe S, Jaster R. 2007. Inhibitory effects of interferon-γ on activation of rat pancreatic stellate cells are mediated by STAT1 and involve down-regulation of CTGF expression. Cellular Signalling 19:782-790.

Flaishon L, Hershkoviz R, Lantner F, Lider O, Alon R, Levo Y, Flavell RA, Shachar I. 2000. Autocrine Secretion of Interferon γ Negatively Regulates Homing of Immature B Cells. The Journal of Experimental Medicine 192:1381-1388.

Fong G, Backman LJ, Hart DA, Danielson P, McCormack B, Scott A. 2013. Substance P enhances collagen remodeling and MMP-3 expression by human tenocytes. Journal of Orthopaedic Research 31:91-98.

Foster W, Li Y, Usas A, Somogyi G, Huard J. 2003. Gamma interferon as an antifibrosis agent in skeletal muscle. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 21:798-804.

Freeland AE, Tucci MA, Barbieri RA, Angel MF, Nick TG. 2002. Biochemical evaluation of serum and flexor tenosynovium in carpal tunnel syndrome. Microsurgery 22:378-385.

Fu S-C, Wong Y-P, Cheuk Y-C, Lee K-M, Chan K-M. 2005. TGF-[beta]1 Reverses the Effects of Matrix Anchorage on the Gene Expression of Decorin and Procollagen Type I in Tendon Fibroblasts. Clinical orthopaedics and related research 431:226-232.

Fu SC, Wang W, Pau HM, Wong YP, Chan KM, Rolf CG. 2002. Increased Expression of Transforming Growth Factor-[beta] 1 in Patellar Tendinosis. Clinical orthopaedics and related research 400:174-183.

Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J. 2001. The use of an antifibrosis agent to improve muscle recovery after laceration. The American journal of sports medicine 29:394-402.

Gallagher S, Heberger JR. 2013. Examining the Interaction of Force and Repetition on Musculoskeletal Disorder Risk A Systematic Literature Review. Human Factors: The Journal of the Human Factors and Ergonomics Society 55:108-124.

Gao HG, Fisher PW, Lambi AG, Wade CK, Barr-Gillespie AE, Popoff SN, Barbe MF. 2013. Increased serum and musculotendinous fibrogenic proteins following persistent low-grade inflammation in a rat model of long-term upper extremity overuse. PloS one 8:e71875.

Germonpre PR, Bullock GR, Lambrecht BN, Van De Velde V, Luyten WH, Joos GF, Pauwels RA. 1999. Presence of substance P and neurokinin 1 receptors in human sputum macrophages and U-937 cells. The European respiratory journal 14:776-782.

Grotendorst GR. 1997. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. Cytokine Growth Factor Rev 8:171-179.

Harrison S, Geppetti P. 2001. Substance P. The International Journal of Biochemistry & Cell Biology 33:555-576.

Heinemeier KM, Olesen JL, Haddad F, Langberg H, Kjaer M, Baldwin KM, Schjerling P. 2007. Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types. J Physiol 582:1303-1316.

Hocevar B, Howe P. 2000. Analysis of TGFβ-Mediated Synthesis of Extracellular Matrix Components. In: Howe P, editor. Transforming Growth Factor-Beta Protocols: Humana Press. p 55-65.

Howard EW, Crider BJ, Updike DL, Bullen EC, Parks EE, Haaksma CJ, Sherry DM, Tomasek JJ. 2012. MMP-2 expression by fibroblasts is suppressed by the myofibroblast phenotype. Exp Cell Res 318:1542-1553.

Hu D, Chen B, Zhu X, Tao K, Tang C, Wang J. 2002. [Substance P up-regulates the TGF-beta 1 mRNA expression of human dermal fibroblasts in vitro]. Zhonghua

zheng xing wai ke za zhi = Zhonghua zhengxing waike zazhi = Chinese journal of plastic surgery 18:234-236.

Igarashi A, Okochi H, Bradham DM, Grotendorst GR. 1993. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Molecular Biology of the Cell 4:637-645.

Ignotz RA, Massagué J. 1986. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. Journal of Biological Chemistry 261:4337-4345.

Ihn H. 2008. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. J Dermatol Sci 49:103-113.

Kant V, Gopal A, Kumar D, Bag S, Kurade NP, Kumar A, Tandan SK, Kumar D. 2013. Topically applied substance P enhanced healing of open excision wound in rats. European journal of pharmacology 715:345-353.

Katayama I, Nishioka K. 1997. Substance P augments fibrogenic cytokine-induced fibroblast proliferation: possible involvement of neuropeptide in tissue fibrosis. J Dermatol Sci 15:201-206.

Kesava Reddy G, Enwemeka CS. 1996. A simplified method for the analysis of hydroxyproline in biological tissues. Clinical Biochemistry 29:225-229.

Kietrys DM, Barr-Gillespie AE, Amin M, Wade CK, Popoff SN, Barbe MF. 2012. Aging contributes to inflammation in upper extremity tendons and declines in forelimb agility in a rat model of upper extremity overuse. PloS one 7:e46954.

Kietrys DM, Barr AE, Barbe MF. 2011. Exposure to repetitive tasks induces motor changes related to skill acquisition and inflammation in rats. J Mot Behav 43:465-476.

Kjaer M. 2004. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiological reviews 84:649-698.

Kjaer M, Langberg H, Heinemeier K, Bayer ML, Hansen M, Holm L, Doessing S, Kongsgaard M, Krogsgaard MR, Magnusson SP. 2009. From mechanical loading

to collagen synthesis, structural changes and function in human tendon. Scand J Med Sci Sports 19:500-510.

Kleiner DM. 1998. Human Tendons: Anatomy, Physiology and Pathology. Journal of Athletic Training 33:185-186.

Koon HW, Shih D, Karagiannides I, Zhao D, Fazelbhoy Z, Hing T, Xu H, Lu B, Gerard N, Pothoulakis C. 2010. Substance P modulates colitis-associated fibrosis. The American journal of pathology 177:2300-2309.

Kumaran C, Shivakumar K. 2002. Calcium- and superoxide anion-mediated mitogenic action of substance P on cardiac fibroblasts.

Lai XN, Wang ZG, Zhu JM, Wang LL. 2003. Effect of substance P on gene expression of transforming growth factor beta-1 and its receptors in rat's fibroblasts. Chinese journal of traumatology = Zhonghua chuang shang za zhi / Chinese Medical Association 6:350-354.

Lambrecht BN, Germonpre PR, Everaert EG, Carro-Muino I, De Veerman M, de Felipe C, Hunt SP, Thielemans K, Joos GF, Pauwels RA. 1999. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. European journal of immunology 29:3815-3825.

Leask A. 2004. Transcriptional profiling of the scleroderma fibroblast reveals a potential role for connective tissue growth factor (CTGF) in pathological fibrosis. The Keio journal of medicine 53:74-77.

Leask A, Abraham DJ. 2004. TGF-beta signaling and the fibrotic response. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 18:816-827.

Leask A, Abraham DJ. 2006. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. Journal of cell science 119:4803-4810.

Li MO, Wan YY, Sanjabi S, Robertson A-KL, Flavell RA. 2006. Transforming growth factor-β regulation of immune responses. Annual Review of Immunology 24:99-146.

Lin TW, Cardenas L, Soslowsky LJ. 2004. Biomechanics of tendon injury and repair. Journal of Biomechanics 37:865-877.

Lipson KE, Wong C, Teng Y, Spong S. 2012. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. Fibrogenesis Tissue Repair 5 Suppl 1:S24.

Luo Q, Song G, Song Y, Xu B, Qin J, Shi Y. 2009. Indirect co-culture with tenocytes promotes proliferation and mRNA expression of tendon/ligament related genes in rat bone marrow mesenchymal stem cells. Cytotechnology 61:1-10.

Messner K, Wei Y, Andersson B, Gillquist J, Rasanen T. 1999. Rat model of Achilles tendon disorder. A pilot study. Cells, tissues, organs 165:30-39.

Mosmann TR, Coffman RL. 1989. TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties. Annual Review of Immunology 7:145-173.

Nakama LH, King KB, Abrahamsson S, Rempel DM. 2006. VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 24:393-400.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. 2004. The role of substance P in inflammatory disease. J Cell Physiol 201:167-180.

O'Kane S, Ferguson MW. 1997. Transforming growth factor βs and wound healing. The international journal of biochemistry & cell biology 29:63-78.

OSHA. 2014. 2014. Prevention of work-related musculoskeletal disorders. In: Occupational Safety & Health Administration USDoL, editor.

Ozturk N, Erin N, Tuzuner S. 2010. Changes in tissue substance P levels in patients with carpal tunnel syndrome. Neurosurgery 67:1655-1660; discussion 1660-1651.

Paparo TSLCRLAA. 1988. Text / Atlas of Histology

Pauly S, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, Wildemann B. 2010. Characterization of tendon cell cultures of the human rotator cuff. Eur Cell Mater 20:84-97.

Perbal B. 2001. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Molecular Pathology 54:57-79.

Perbal B. 2004. CCN proteins: multifunctional signalling regulators. The Lancet 363:62-64.

Phanish MK, Winn SK, Dockrell MEC. 2010. Connective Tissue Growth Factor-(CTGF, CCN2) – A Marker, Mediator and Therapeutic Target for Renal Fibrosis. Nephron Experimental Nephrology 114:e83-e92.

Piligian G, Herbert R, Hearns M, Dropkin J, Landsbergis P, Cherniack M. 2000. Evaluation and management of chronic work-related musculoskeletal disorders of the distal upper extremity. Am J Ind Med 37:75-93.

Poppe S. 2010. Establishment and investigation of tendon-derived cell lines immortalized by the human telomerase reverse transcriptase gene. In.

Rani S, Barbe MF, Barr AE, Litvin J. 2009. Periostin-like-factor and Periostin in an animal model of work-related musculoskeletal disorder. Bone 44:502-512.

Rechardt M, Shiri R, Matikainen S, Viikari-Juntura E, Karppinen J, Alenius H. 2011. Soluble IL-1RII and IL-18 are associated with incipient upper extremity soft tissue disorders. Cytokine 54:149-153.

Rempel DM, Diao E. 2004. Entrapment neuropathies: pathophysiology and pathogenesis. Journal of electromyography and kinesiology : official journal of the International Society of Electrophysiological Kinesiology 14:71-75.

Riley G. 2004. The pathogenesis of tendinopathy. A molecular perspective. Rheumatology 43:131-142.

Riley G. 2008. Tendinopathy--from basic science to treatment. Nature clinical practice Rheumatology 4:82-89.

Ross MH KE, Romrell LJ. 1989. Connective tissue, in Histology: A Text and Atlas. Second edition.: pp85 –116.

Schild C, Trueb B. 2002. Mechanical Stress Is Required for High-Level Expression of Connective Tissue Growth Factor. Experimental Cell Research 274:83-91.

Seher A, Nickel J, Mueller TD, Kneitz S, Gebhardt S, ter Vehn TM, Schlunck G, Sebald W. 2011. Gene expression profiling of connective tissue growth factor (CTGF) stimulated primary human tenon fibroblasts reveals an inflammatory and wound healing response in vitro. Mol Vis 17:53-62.

Seluanov A, Vaidya A, Gorbunova V. 2010. Establishing Primary Adult Fibroblast Cultures From Rodents.e2033.

Shamis Y, Hewitt KJ, Carlson MW, Margvelashvilli M, Dong S, Kuo CK, Daheron L, Egles C, Garlick JA. 2011. Fibroblasts derived from human embryonic stem cells direct development and repair of 3D human skin equivalents. Stem Cell Research & Therapy 2:10-10.

Shi Y, Massagué J. 2003. Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. Cell 113:685-700.

Smith CA, Stauber F, Waters C, Alway SE, Stauber WT. 2007. Transforming growth factor-beta following skeletal muscle strain injury in rats. J Appl Physiol 102:755-761.

Sobral LM, Montan PF, Martelli-Junior H, Graner E, Coletta RD. 2007. Opposite effects of TGF-beta1 and IFN-gamma on transdifferentiation of myofibroblast in human gingival cell cultures. Journal of clinical periodontology 34:397-406.

Song JJ, Aswad R, Kanaan RA, Rico MC, Owen TA, Barbe MF, Safadi FF, Popoff SN. 2007. Connective tissue growth factor (CTGF) acts as a downstream mediator of TGF-beta1 to induce mesenchymal cell condensation. J Cell Physiol 210:398-410.

Sorrell JM, Caplan AI. 2004. Fibroblast heterogeneity: more than skin deep. Journal of cell science 117:667-675.

Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. 2014. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiological reviews 94:265-301.

Terada S, Ota S, Kobayashi M, Kobayashi T, Mifune Y, Takayama K, Witt M, Vadalà G, Oyster N, Otsuka T, Fu FH, Huard J. 2013. Use of an Antifibrotic Agent Improves the Effect of Platelet-Rich Plasma on Muscle Healing After Injury.

Thomopoulos S, Parks WC, Rifkin DB, Derwin KA. 2015. Mechanisms of tendon injury and repair. Journal of Orthopaedic Research:n/a-n/a.

Toyoda M, Makino T, Kagoura M, Morohashi M. 2000. Immunolocalization of substance P in human skin mast cells. Arch Dermatol Res 292:418-421.

Tzortzaki EG, Antoniou KM, Zervou MI, Lambiri I, Koutsopoulos A, Tzanakis N, Plataki M, Maltezakis G, Bouros D, Siafakas NM. 2007. Effects of antifibrotic agents on TGF-beta1, CTGF and IFN-gamma expression in patients with idiopathic pulmonary fibrosis. Respiratory medicine 101:1821-1829.

van Boxel-Dezaire A, Stark G. 2007. Cell type-specific signaling in response to interferon-γ. In: Interferon: The 50th Anniversary: Springer. p 119-154.

Wang Q, Usinger W, Nichols B, Gray J, Xu L, Seeley T, Brenner M, Guo G, Zhang W, Oliver N, Lin A, Yeowell D. 2011. Cooperative interaction of CTGF and TGF-beta in animal models of fibrotic disease. Fibrogenesis Tissue Repair 4:4.

Wen F-Q, Liu X, Kobayashi T, Abe S, Fang Q, Kohyama T, Ertl R, Terasaki Y, Manouilova L, Rennard SI. 2004. Interferon-γ inhibits transforming growth factor-β production in human airway epithelial cells by targeting Smads. American journal of respiratory cell and molecular biology 30:816-822.

Wynn TA. 2003. IL-13 Effector functions*. Annual Review of Immunology 21:425-456.

Wynn TA. 2007. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. The Journal of clinical investigation 117:524-529.

Yao Y, Zhang J, Tan D-Q, Chen X-Y, Ye D-F, Peng J-P, Li J-T, Zheng Y-Q, Fang L, Li Y-K, Fan M-X. 2011. Interferon-γ Improves Renal Interstitial Fibrosis and

Decreases Intrarenal Vascular Resistance of Hydronephrosis in an Animal Model. Urology 77:761.e768-761.e713.

Yaraee R, Ghazanfari T. 2009. Substance P Potentiates TGFß-1 Production in Lung Epithelial Cell Lines. Iranian Journal of Allergy, Asthma and Immunology 8:19-24.

Ziesche R, Hofbauer E, Wittmann K, Petkov V, Block LH. 1999. A preliminary study of long-term treatment with interferon gamma-1b and low-dose prednisolone in patients with idiopathic pulmonary fibrosis. The New England journal of medicine 341:1264-1269.

Zubrzycka M, Janecka A. 2000. Substance P: transmitter of nociception (Minireview). Endocrine regulations 34:195-202.

APPENDIX A

TENOCYTE ISOLATION AND CULTURE

Materials:

Filter System (Corning; 430186)

Cell Culture Dish (Corning; 430165)

Collagenase (Clostridopeptidase A, C-0130 Sigma)

D-MEM (Cellogro; 10-013-CV;500ml)

L-Glutamine (Cellogro; 25-005-Cl; 100ml)

Heat-inactivated FBS (Gibco; 16140-063; 100ml)

Antibiotic-Antimycotic (Gibco; 15240-062; 100ml)

Methods:

1. Sterilize autoclaved scissors and forceps with 70% ethanol.
2. Prepare collagenase (clostridopeptidase A; 2mg/ml) diluted in D-MEM + 1% Antibiotic / Antimycotic, make 500 µL aliquots and freeze at -20°C. Thaw a new aliquot before every use. The solution may appear cloudy after thawing. Vortex the solution until it becomes clear.
3. Isolate tenocytes from the flexor digitorum tendons obtained from normal young adult female rats. Put tendons into a petri dish with 1X HBSS + 1% Antibiotic-Antimycotic (A/A); separate muscles from tendons using sterile scalpels and scissors (on ice).
4. Dissect tendons carefully and mince with scissors in 1X HBSS + 1% A/A.
5. Discard HBSS, wash tissue fragments with D-MEM + 1% A/A three times.

6. Transfer tissue fragments into a 100mm tissue culture dish using a sterile scalpel.
7. Cut tissue into ~3 mm pieces using two scalpels. Use two blades and a scissor action, starting from the center and pulling apart. Keep tissue balled up, do not cut piece by piece.
8. Transfer minced fragments into a sterile 15 mL tube containing 5 mL of D-MEM+ 1% A/A. The minced fragments will be enzymatically digested at 37ºC using collagenase (clostridopeptidase A; 2mg/ml) diluted in D-MEM, check digestion after 30 min, and then every 10 min but no longer than 60 minutes in total. Do not over-digest tissue.
9. Pipet solution with digested tissue fragments up and down to break clumps. Transfer the solution to a sterile 50 ml tube. Mix by inversion a few times.
10. Centrifuge digested product at 800 g for 5 min.
11. Discard supernatant and re-suspend pellet with 4 mL D-MEM + 10% FBS + 1% A/A; pipet suspension with maximum force to break tissue pieces. Culture the suspension in a 35mm cell-culture-treated petri dish with D-MEM supplemented with 10% heat-inactivated FBS, 1% A/A and 2mM L-glutamine at 37℃ and 5% CO2.
12. Next day, aid dissociation of any large pieces of tissue by titrating with a sterile pipette.
13. Maintain sterility. Continue incubating tissue for 3 days at 37℃ and 5% CO2 to allow cells to adhere and spread away from tissues.

14. Adherent cells are usually sparse, and a few larger chunks remain. Remove tissue chunks to a new cell culture dish. Feed cells in the original plate with fresh media, and add media to the new dish with the chunks. Continue incubating cells.
15. Check plates every day for fibroblasts and media color. If media changes color to yellow, potential contamination or overcrowding of cells might occur.
16. Incubate cells and tissue fragments for an additional 7 days, all viable fibroblasts have exited tissue fragments.
17. Change the media every 3-4 days. Cells will be maintained in 35mm cell-culture-treated petri dishes at 37 ℃ and 5% CO_2 until the cells reach 80-90% confluence.
18. Harvest the cells. Cells are counted and plated in a T75 flask with D-MEM media + 10% FBS + 1% A/A.
19. These cultures will be expanded and cells from passages 3 to 6 will be used.

APPENDIX B

WESTERN BLOT PROTOCOL

Materials:

Laemmli loading buffer (4X): #161-0747

Anti-CCN2 antibody: #sc-14939

Anti- TGFß-1 antibody: #sc-146

Methods:

SDS-PAGE

1. Solutions for Tris Glycine SDS-Polyacrylamide Gel Electrophoresis

10% SDS gel	10 ml	STACK	3 ml
H2O	4.0	H2O	2.1
30% Acrylamide mix	3.3	30% Acrylamide mix	0.5
1.5 M Tris (PH 8.8)	2.5	1.5 M Tris (PH 8.8)	0.38
10% SDS	0.1	10% SDS	0.03
10% APS	0.1	10% APS	0.03
TEMED	0.004	TEMED	0.003

2. Boil samples for 5 min at 100 C°.

3. Load samples (50 µg each lane) to the gel.

4. Run the gel at 80V, allow migration to continue until blue dye front is at the end of the glass plate, but has not migrated off the gel.

Transferring

1. Prepare the transfer sandwich on the black panel in the tray filled with transfer buffer.

a. One spongy

b. Filter paper

c. SDS gel

d. nitrocellulose membrane

e. Filter paper

f. Spongy

Note: Remove air bubbles by rolling a glass tube on the membrane.

2. Cover the sandwich with the clear panel, fasten with the latch, and insert the gel cassette into the electrode module with the black panel facing the black cathode electrode panel.

3. Fill the chamber with transfer buffer, transfer for 75 minutes at 100V.

Notes: keep the box in ice. Transfer the membrane in cold.

Blocking and incubating

1. Incubate the membranes with Blocking Buffer for an hour at room temperature with gentle shaking.

Blocking buffer: 5% non-fat milk in TBS + 0.1% Tween 20.

2. Dilute primary antibody in 5% non-fat milk in TBS + 0.1% Tween 20 and incubate the membrane with the diluted primary antibody with gentle shaking overnight at 4 C°.

3. Wash the membrane 3 times, 5 minutes each at room temperature in TBS + 0.1% Tween 20 with gentle shaking, using a generous amount of buffer.

4. Dilute secondary antibody in 5% non-fat milk in TBS + 0.1% Tween 20 and incubate the membrane with the diluted secondary antibody with gentle shaking for an hour at room temperature. Protect from light during incubation.

5. Wash the membrane 3 times, 5 minutes each at room temperature in TBS + 0.1% Tween 20 with gentle shaking, using a generous amount of buffer. Protect from light during washes.

6. The membrane is now ready to image, with a Li-Cor Odyssey Infrared Imaging System.

APPENDIX C

HYDROXYPROLINE ASSAY OF CELL LYSATES

Materials and Methods

Chemicals: 4-(Dimethylamino) benzaldehyde and trans-4-Hydroxy-L-proline were purchased from Sigma Chemical. Chloramine-T Trihydrate, Sodium acetate, citric acid monohydrate, perchloric acid, n-propanol, sodium hydroxide and acetic acid were purchased from Fisher Scientific. All other chemicals were of analytical grade.

Preparation of Reagents

Hydroxyproline stock

A solution containing 1 mg/mL of Hydroxyproline was prepared in distilled water.

Acetate-citrate buffer PH 6.5

The buffer was prepared by dissolving 120 g of sodium acetate trihydrate, 50.1 g of citric acid monohydrate, 12 mL acetic acid, and 34 g of sodium hydroxide in distilled water; pH was adjusted to 6.5 and brought to one liter.

Chloromine T reagent (0.056M)

1.27 g of chloromine T Trihydrate was dissolved in 10 mL n-propanol and brought to 100ml with acetate-citrate buffer.

Ehrlich's reagent (1M)

1.5 g 4-(Dimethylamino) benzaldehyde was dissolved in 6mL n-propanol and 2.6ml 70% perchloric acid and brought to 10ml with ddH2O. Since this reagent is not stable, it was freshly prepared.

Assay procedure

All samples and standards were run in triplicate. Use ddH2O for the preparation of standards and samples.

Hydroxyproline standards for chlorimetric reaction

Dilute 10 µL of the 1 mg/mL Hydroxyproline Standard Solution with 90 µL of water to prepare a 0.1 mg/mL standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 0.1 mg/mL hydroxyproline standard solution into a 96 well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 µg/well standards.

Sample preparation

Homogenize cells in 100µL of water and transfer to an eppendorf tube. Add 100 µL of concentrated hydrochloric acid (HCL, ~12M), cap tightly, and hydrolyze at 120℃ for at least 3 hours. Transfer 50 µL of supernatant to a 96well plate.

Evaporate all wells to dryness in a 60 °C oven.

Assay:

1. Add 100 µL of the Chloromine T/Oxidation Buffer Mixture to each sample and standard well. Incubate at room temperature for 20 min.

2. Add 100 µL of the Ehrlich's reagent (1M) to each sample and standard well, and incubate for 90 minutes at 60℃.

3. Measure absorbance at 560nM (A_{560}).

APPENDIX D

CYTOSPIN PROTOCOL

Procedure, cell preparation

1. Harvest cells and wash them twice in PBS using centrifugation (1500 rpm for 5 min) to remove residual protein.

2. Adjust the cell concentration to 4–5 x 10^5 cells per mL in PBS.

3. Assemble the CytoSpin centrifuge's sample chamber, filter card, slide, and racks according to the manufacturer's instructions.

4. Load 100 µL of cells in each sample chamber.

5. Centrifuge the slides at 600 rpm for 2 min.

6. Add 100 µL 4% paraformaldehyde in 1x PBS in each sample chamber immediately, and centrifuge the slides at 600 rpm for 2 minutes.

7. Remove the slides from the rack and place them on a staining rack.

8. Continue with the staining procedure.

APPENDIX E

IMMUNOCYTOCHEMISTRY FOR CCN2 ON CHAMBER SLIDES

1. Seed cells on 8-well chamber slides (LabTek, #177445) at a density of 2,000 cells/well.
2. Serum deprivation (1% FBS) for 24 h.
3. Treat cells with a standard concentration of 100 nM of SubP (Tocris; 1156) or 10 ng/ml of TGF-β1 (EMD Millipore; GF111) alone or in combination for a 48 h period.
4. Fix cultured cells with 4% paraformaldehyde in 1x PBS for 20 minutes at room temperature.
5. Wash twice with 1x wash buffer (0.1% Tween-20 in 1x PBS).
6. Permeabilize cells with 0.1% Triton X-100 in 1x PBS for 5 minutes at room temperature.
7. Wash twice with 1x wash buffer (0.1% Tween-20 in 1x PBS).
8. Apply blocking solution (3% BSA in 1x PBS) for 30 minutes at room temperature.
9. Dilute primary antibody (Anti-CCN2; Santa Cruz, SC-14939) to a working concentration (1:50) in blocking solution, and incubate at 4 ℃ overnight.
10. Wash three times (5-10 minutes each) with 1x wash buffer.
11. Dilute secondary antibody (Cy2, DyLight 488, Cy3, or DyLight 594) (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:500 each in PBS for 60 min at room temperature.
12. Wash three times (5-10 minutes each) with 1x wash buffer.

13. OPTIONAL: Following this washing step, nuclei counterstaining can be performed by incubating cells with DAPI (dilute in 1x PBS at a concentration of 1:500) for 1-5 minutes at room temperature, followed by washing cells three times (5-10 minutes each) with 1x wash buffer.
14. Mount the cells in 80% glycerol in 1x PBS, and coverslip them.
15. Fluorescence images can be visualized with a fluorescence microscope.
16. NOTE: Be sure to use the correct filter for visualizing fluorescent-labeled cells.

The percentage area of immunostaing for CCN2 was quantified using an image analysis system (BioQuant Osteo, BioQuant, Nashville, TN).