The Role of ATP in Tendon Response to Stress

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ABSTRACT

ANN MARIE FOX: The Role of ATP in Tendon Response to Stress
(Under the direction of Albert J. Banes, Ph.D.)

Tendon cells coordinate activities such as force transmission, cell division, and matrix remodeling to maintain tissue function and integrity. This is especially evident in the response to high mechanical load demands. Signaling to load and the subsequent feedback on gap junction expression and function are likely modulated by ATP, mechanical load, and other mediators. Tenocytes secrete ATP through connexin hemichannels as well as the cystic fibrosis transmembrane regulator channel capable of nucleotide release in tendon cells. The primary goal for this dissertaiton was to define the intersecting pathways of ATP-mediated intercellular signaling in tendons responding to stress.

Use of transgenic mice with specific purinoceptor deficits were used to study tenocyte responses to mechanical load. Data supports that stimulation of ATP-activated $P2Y_2$ receptors may amplify a load signal while activation of a $P2Y_1$ purinoreceptor through ADP may dampen a load signal. *Wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) cells were subjected to mechanical indentation with a micropipette as well as pharmacological adds to examine select downstream response variations that highlight the interplay between $P2Y_1$ and $P2Y_2$ purinoreceptors. The studies help to unravel the complex and interdependent pathways that regulate responses to load. To investigate the downstream consequences of the altered response

communication, mechanical testing of *wt* and knockout mouse tendons and detailed microscopy studies were performed. This demonstrated the mechanical implications attributable to the omitted ATP and ADP receptors, demonstrating a markedly decreased strength in $P2Y_1$(-/-) tendons. Finally, immunohistochemistry was performed to elucidate the role of connexins in the altered signaling response of purinoreceptor null tenocytes. Images show a both punctate and diffuse cytosolic staining in all cells. However, the degree of cytosolic staining in $P2Y_2$(-/-) cells indicates a potential gap junctional assembly or trafficking problem.

Through results of this dissertation, the scientific community will gain a better understanding of the importance of purinoreceptors in the mechanical sensitivity of tendon cells. The information resulting from these experiments may also open doors for pharmaceutical therapy for damaged or deteriorating tendons and will enhance our knowledge of purine receptor interplay that is essential for *in vivo* maintenance and *ex vivo* production of tendon tissue.

DEDICATION

To all my family and friends, for their loving support shown in every way

especially,

to Chris, for his unconditional love and daily encouragement;

and

to my parents, Tom and Colleen, for providing me the best possible foundation for success – our loving family.

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LIST OF ABBREVIATIONS AND SYMBOLS

$[Ca^{2+}]_{ic}$	Intracellular calcium concentration
3D	Three dimensional
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATP-γs	Adenosine 5'-O-(3-thiotriphosphate)
ADP	Adenosine diphosphate
ADP-βs	Adenosine 5'-O-(2-thiodiphosphate);
AMP	Adenosine monophosphate
BAT(s)	Bioartificial Tendon(s)
Ca^{2+}	Calcium
Cl^-	Chloride
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane regulator
COX	Cyclooxygenase
Cx	Connexin
DAG	1,2-diacylgylcerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
E	Young's modulus
EBSS	Earles' Balanced Salt Solution
Ecto-NTPases	Ecto-5'-nucleotidases

EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
Fura-2AM	Fura –2 acetoxy methylester
GAG	Glycoaminoglycans
GPCR	G-protein coupled receptor
IGF	Insulin- like growth factor
IL-1β	Interleukin-1 beta
IP_3	Inositol (1,4,5)-trisphosphate
Ko	Knockout
l	Length
mA	Milliamp
ml	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
P2Y	Purinoreceptor type Y
$P2Y_1$(-/-)	$P2Y_1$ purinoreceptor knockout
$P2Y_2$(-/-)	$P2Y_2$ purinoreceptor knockout
P2X	Purinoreceptor type X
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E2

PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PSF	Penicillin, Streptomycin, Fungicide Antibiotic/mitotic
RNA	Ribonucleic acid
RT	Reverse transcriptase
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SE	Standard error
TGF-β	Transforming growth factor-beta
Wt	Wild type

Greek Symbols

ε	Strain
σ	Stress
µl	Microliter
µm	Micrometer

1 Introduction

1.1 Background

Cellular communication is one of the most elemental requirements for tissues. In a mechanically active tissue such as tendon, intercellular signaling is necessary to preserve tissue homeostasis. Nucleotides are powerful mediators of the load response, and are a potential candidate for involvement in tendinopathy. Receptors for these molecules, called purinoreceptors, are therefore targets for understanding the responsibilities that nucleotides hold as part of the mechanotransduction pathway.

Tendon is a dense fibrous connective tissue, consisting of relatively few fibroblasts within a collagen-rich extracellular matrix. Cells within the tendon matrix, called tenocytes, are capable of synthesizing and degrading the tendon matrix. This process is vital to the tissue response to exercise, mechanical strain, and injury (Woo et al, 1982; Humble et al., 2001; Gimbel et al., 2007; Westh et al., 2007; Banes et al., 2007). Tendon injuries and chronic tendon pain are extremely common in athletes, as well as in the general population. Despite their prevalence, these conditions are poorly understood and often do not respond well to treatment.

Calcium is a ubiquitous second messenger used by virtually every cell type. This signaling molecule is involved at some stage in a gamut of cellular processes

necessary for the initiation, maintenance, and termination of life. Elevated calcium levels spread from cell to cell, in what has been termed an intercellular calcium wave, and has been studied in a variety of cells including tenocytes (Banes et al., 1995) osteoblasts (Jorgensen et al., 1997), and epithelial cells (Sanderson et al., 1990). These signals can propagate through one or both of these two distinct cascades: a.) relying on the diffusion of IP_3 through gap junctions that physically and electrically couple neighboring cells, initiating the spread of a calcium wave, and b.) through the paracrine action of nucleotides, which bind to purinergic receptors (purinoreceptors) that are coupled to PLC, leading to the generation of IP_3 (Berridge et al., 1993; Frame et al., 1997; Saur et al., 2000, Homolya et al., 2000).

Nucleotides are powerful signaling molecules that are released in response to cellular damage, and are also important modulators of cell growth, ion transport, and inflammation (Erlinge, 1998; McCoy et al., 1999; Dixon et al., 1999). The precise magnitude, level, and frequency of stimuli necessary to maintain tissue homeostasis is not currently known. However, it is very likely that an abnormal level of any of the aforementioned may have a role in the pathogenesis of tendinopathy (Jozsa & Kannus, 1997; Dixon et al., 1999).

Tendon cells, which express both gap junctions and purinoceptors, respond to mechanical load by increasing intracellular calcium ($[Ca^{2+}]_{ic}$). Purinoreceptors and mechanical stimulation both increase $[Ca^{2+}]_{ic}$ and may share a common pathway. Therefore cells lacking any or all of these components should have altered mechanosensitivity. Thus, it is essential to understand the part each actor plays in

the process of mechanotransduction in tendon to work toward effective prevention and treatment of tendon injury and disease.

1.2 Project Overview

The major goal of this dissertation was to elucidate the role of $P2Y_1$ and $P2Y_2$ purinoreceptors in response to mechanical stimulation, as well as the downstream effects caused by response variations. On a daily basis, tendon cells coordinate activities such as force transmission, cell division, and matrix remodeling to maintain tissue function and integrity. This is especially evident in the response to high mechanical load demands (Banes et al., 1995). These cells are widely dispersed throughout the collagen fibers, but have been shown to communicate via gap junctions at the end of long cellular processes (McNeilly et al., 1996, Wall et al., 2007). Signaling to load and the subsequent feedback on gap junction expression and function are likely modulated by ATP, mechanical load, and other mediators. ATP is widely recognized as an important molecular messenger for cell-cell communication (Ralevic & Burnstock, 1998). Tenocytes secrete ATP through connexin hemichannels, the cystic fibrosis transmembrane regulator (CFTR) channel capable of nucleotide release in tendon cells, as well as $P2X_7$ and the connexin-related protein pannexin1 (Schwiebert et al., 1999; Beigi et al., 1999; Cotrina et al., 1998; Locovei et al., 2006; Schneider et al., 2006; Eltzschig et al., 2006). Tenocytes react to ATP by increasing intracellular calcium levels and modulating MMP and COX2 expression (Tsuzaki et al., 2003). ATP modulates load and inflammatory responses in tenocytes and is a candidate for involvement in tendinopathies. High concentrations of ATP and its metabolites likely occur in

tendon tissue during trauma since lysed cells and platelets release milliMolar quantities of ATP which, when degraded by ecto-NTPases, produce ADP, AMP, and adenosine (Zimmerman et al., 2000; Tsuzaki et al., 2003, 2005). Use of transgenic mice with specific purinoceptor deficits are useful in studying tenocyte responses to mechanical load, and investigating the structural disparities resulting from the absence of each purinoreceptor. The primary goal for this research is to define the intersecting pathways of ATP-mediated intercellular signaling in tendon response to stress, and correlate those changes to the structural integrity of tendon tissue.

We have outlined a research plan that tests the fundamental hypothesis that ATP magnifies a load response and ADP modulates and dampens the mechanosensitivity of tendon cells. The role of gap junctions and purinoreceptor interaction will be tested using tendon cells grown in cultures and frozen tissue immunohistochemistry. *Wt,* $P2Y_1$(-/-), and $P2Y_2$(-/-) cells were subjected to mechanical indentation with a micropipette to elucidate the communication behavior when either $P2Y_1$ or $P2Y_2$ purinoreceptors are absent. In addition, mechanical testing of whole tendon provided insight into the downstream consequences of response variations within $P2Y_1$ and $P2Y_2$ knockout mice tissues. Moreover, these studies examined selected response patterns that highlight the interplay between $P2Y_1$ and $P2Y_2$ purinoreceptors, connexin 43, and intercellular calcium transients.

Studies involving cells from purinoceptor knockout mice can help unravel the complex and interdependent pathways that regulate responses to load, and reveal how nucleotides and gap junctions modulate wound healing. The outcomes of this research contribute to the development of a functional tissue engineered

bioartificial tendon, as well as pharmaceutical therapy for damaged or deteriorating tendons.

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2 Tendon Structure and Function

2.1 The structural hierarchy of tendon

The tendon is a mechanically impressive tissue; its role in the skeletal system is two fold: to transmit tensile loads generated by striated skeletal muscle to bone to permit ambulation and to stabilize joints. (Woo, 2000; Lo, 2002) The unique structural hierarchy of tendon imparts specific material properties that enable the tissue to withstand such forces as tension, compression, and shear during normal daily activities (Cotrina et al., 1998; Lazarowski et al., 1997; Tsuzaki et al., 2004; Dixon et al., 2000; Weibe et al., 1999). The seminal work of Kastelic in 1967 highlighted the hierarchal structure of tendon. Additional contributions over the years have lead to the discernment of the syncytial nature of tenocytes in tendon, as well as the importance of gap junctions and purinoreceptors in maintaining this structure (McNeilly et al., 1996; Banes et al., 1995, 2007).

Tendons are not a uniform structure. Instead, they vary in their physiology and biochemistry according to the environments of each anatomic location, and even along their length (Jozsa & Kannus 1997). Generally, however, they share the same macroscopic structure (Figure 2.1). A thin sheet of fibrillar connective tissue, called endotenon, encases groups of collagen fibers to form the primary fiber bundle, or subfascicle. This provides a conduit for a delicate vasculature system to provide nutrients to the tissue, as well as innervation and lymphatics (Fenwick et al., 2002).

Subfascicles are bundled into secondary bundles, or fascicles, again sheathed with endotenon. Proteoglycans lie between this layer of endotenon and the fascicle. The tertiary bundle is formed by groupings of fascicles, and finally these collectively form the tendon. The tendon is surrounded by a delicate layer of connective tissue called the epitenon. This sleeve provides a low-friction membrane so tendons can glide over other tendons, bursas, and pulleys within the anatomy of the joint (Figure 2.2).

The tendon has a complex organization of tenocytes: fibroblast cells with spindle-like nuclei and sparse cytoplasm, surrounding the collagen bundles inside tendon as well as at the tendon surface. Internal cells may act to maintain collagen in tendon. External cells in the epitenon may provide lubrication for tendon gliding. (Banes et al., 1988) These cells produce and maintain the proteins that fabricate the complex structure responsible for the tissue's specialized properties. Tenocytes migrate between collagen fibers within the fascicles of tendon. Also, tenocytes can generate their own traction forces as they remodel the matrix, as seen in 3D linear gel constructs populated with tenocytes (Banes et al., 2003). These cells receive nutrients and expel waste through the vasculature system within the tendon. Blood vessels are normally seen throughout the endotenon and paratenon; however, many regions of the tendon are poorly vascularized.

The ends of the tendon are called the osseotendinous junction (OTJ) and the myotendinous junction (MTJ), where the tendon joins the bone and muscle, respectively. These are highly specialized regions where the tension generated by the muscle fibers can be propagated through the tendon to the bone. The interdigitation of the tendon into the muscle body is part of a complex architecture

that is essential for the force transmission involved in ambulation. At the OTJ collagen fibril orientation is random and presents with both mineralized and unmineralized fibrocartilage (Clark & Stechschulte, 1998). These two regions present the most complicated feat of creating bioartificial tendons. Nature has evolved into a near perfect integration between the tendon and bone and the tendon and muscle. The challenge for tissue engineering will be to mimic these properties to achieve successful attachment of the bioartificial tissues.

2.2 Tendon material properties

Tendons demonstrate high mechanical strength, flexibility, and optimal elasticity to perform their unique role in the body (Kirkendall et al., 1997). Connective tissues, including tendon, are viscoelastic materials. As the name implies, they exhibit properties of both viscous and elastic materials. Viscous materials resist shear flow and strain linearly when a stress is applied, absorbing almost all of the energy. Elastic materials, in contrast, strain instantaneously when loaded, and return to their native state once the strain is removed. They are not able to absorb the energy as viscous materials do, but instead dissipate the energy. Viscoelastic materials have elements of both, and these properties in tendon are under scrutiny as the structural elements important to its function are studied.

An important consideration is that viscoelastic materials exhibit time-dependent strain behavior. A useful way to characterize materials is by looking at a stress/strain (σ/ε) curve (Figure 2.3). Data are collected with tensile load applying instrumentation that allows extension and force to be tracked simultaneously. The

outputs are plotted on a curve as strain (the change in length (l_1/l_0)), and engineering stress (force/undeformed_area). Many viscoelastic polymers and biological materials, including tendon, will initially experience large strains for relatively low stresses. However, as the strain increases, the material will become stiffer and more difficult to strain. This results in a J-shaped curve that can be broken into several phases. The first part, called the “toe region”, results from the large extensions for small stresses and a low shear modulus, usually from strains of 0-3%. As the force increases, the stiffness of the material rises as well, and large stresses result in strains of 2-5%. The modulus of the material (E) can be derived from this linear portion of the graph. When strains rise above 4%, the material enters a yield region where permanent (plastic) deformation can occur. Ultimate failure in tendon occurs at strains of 5-15%. (Ozkaya & Nordin, 1999)

2.3 The origins of tendon strength

Hierarchical solids contain structural elements which themselves have structure; this is true of tendon. The mechanisms through which tendon strength is developed, however, are not fully understood. The Achilles tendon is subjected to the highest loads in the body, with tensile loads up to ten times body weight during running, jumping, hopping, and skipping. (O’Brien, 2005) This impressive behavior reflects the material’s chemistry and microstructure. In a composite material like tendon, for example, the collagen fibers provide the strength and stiffness of the material (Robinson et al., 2005). They are not initially loaded, however, and there is a lag between application of stress and tensing the main structural component of the

material, as shown by the toe region of the graph (Figure 2.3). It is not until the fibers align with the load that a transition to the plastic phase of the graph occurs. Thus, characteristics related to collagen such as total collagen content, arrangement of individual collagen molecules into microfibrils, subfibrils, fibrils, and fascicles, collagen fibril size, area fraction, and numerical density are all important determinants of tendon strength (Battaglia et al., 2003). Inter- and intramolecular covalent bonds called crosslinks within the tissue ultimately determine the mechanical behavior of collagen (Sharma, 2005).

Biochemical measures of tendon strength have included amount and type of collagens, with an emphasis on increased expression of the principle structural protein, type 1 collagen, proteoglycans (especially decorin or fibromodulin that regulate fibril diameter, or biglycan to resist compression) (Vogel, 1989). The types of collagen crosslinks are also important to structural integrity, both those within (aldol crosslinks) and between (reducible crosslinks such as dihydroxylysinonorleucine) collagen triple helices (Katz et al., 1982; Banes et al., 1983).

Collagen fibrils are the most elemental part of the tendon. Fibrils are assembled into fibers, held together by crosslinks formed between soluble tropocollagen molecules among the fibrils (Jozsa & Kannus, 1997). These crosslinks provide mechanical stiffness as well as resistance to breakdown by proteases, which contribute to the long half-life of collagen in tendon (Bensusan, 1972). Collagen fibers align with the direction of load, which provides high resistance to tensile forces.

Type I collagen is the major structural element in tendon (70-86% of dry weight) while collagen III, V, VI , XII, and XIV are present in much lower levels (5% of dry weight). Elastin (2-3% of dry weight) contributes to the energy storing capacity in tendons, while proteoglycans such as decorin, biglycan, fibromodulin, and aggrecan (1-5% of dry weight), which bind the collagen fibers together, are gaining importance in structural roles. Water comprises 50-75% of the wet weight of the tissue, demonstrating the hydrophilic nature of its constituents.

Tendons have an amazing ability to adapt to the demands of their mechanical environment. Sensory apparati found within tendon include Golgi tendon organs, that detect changes in muscle tension and pressure, Vater-Pacini and Ruffini corpuscles act as motion sensors, and free nerve endings serve as nocioceptors. (Jozsa & Kannus, 1997) Collagen types II, IX, X, and XI are present in fibrocartilagenous zones where tendon is subjected to shear forces or compression. (Fukuta et al., 1998; Waggett et al., 1998) Recent research has emphasized the importance in the amount and relative distribution of various proteoglycans, such as decorin, as well as the degree of hydration and collagen cross-linking. (Iozzo, 1998; Derwin et al., 2001; Robinson et al., 2005) Each of these modifications allows the tendon to perform a remarkable load-transferring function.

2.4 Conclusions

Tendons are a mechanically important tissue within the musculoskeletal system. A unique molecular architecture provides tendons with impressive mechanical properties, necessary to effect ambulation. Many factors can affect the

balance of these elements, reinforcing the need to better understand the complex interactions that maintain tendon structure.

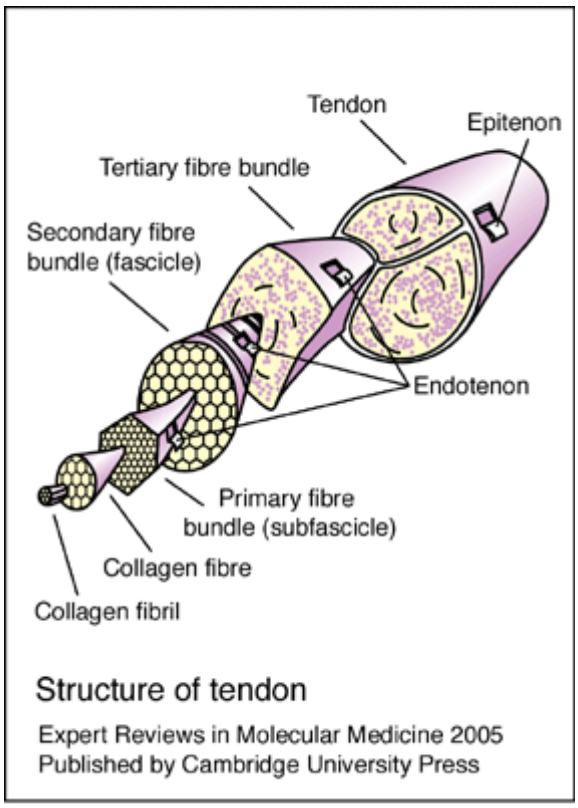

Figure 2.1 Tendon consists of a highly ordered hierarchy of successively larger structural units that contribute to its mechanical properties.

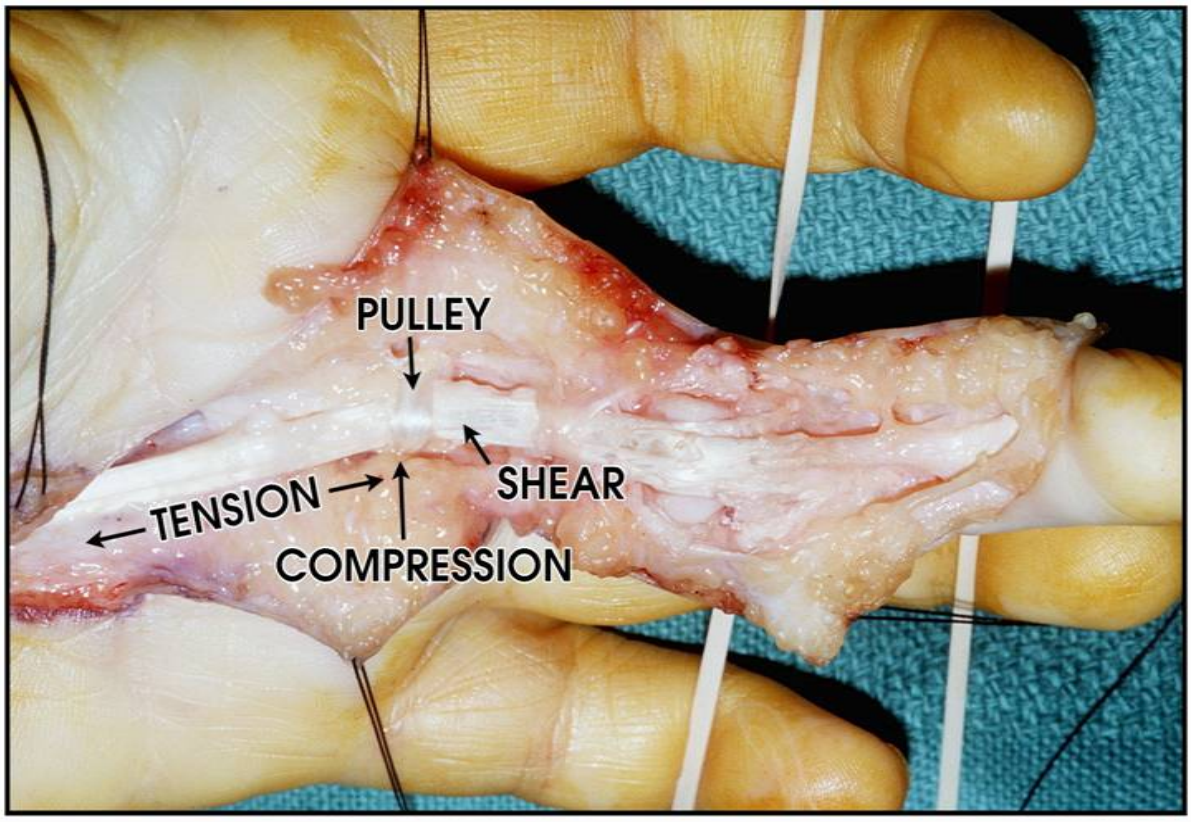

Figure 2.2 The material properties of a tendon vary along its length so that the tissue can withstand the application of differing forces. Shown here, a human flexor digitorum profundus tendon in the third digit of the hand. In addition, vessels, nerves and component cells contend with the shear created during excursion, compression where tendons travel through soft tissue pulleys or over bone, and tensile forces transferred from muscle to the tendon matrix.

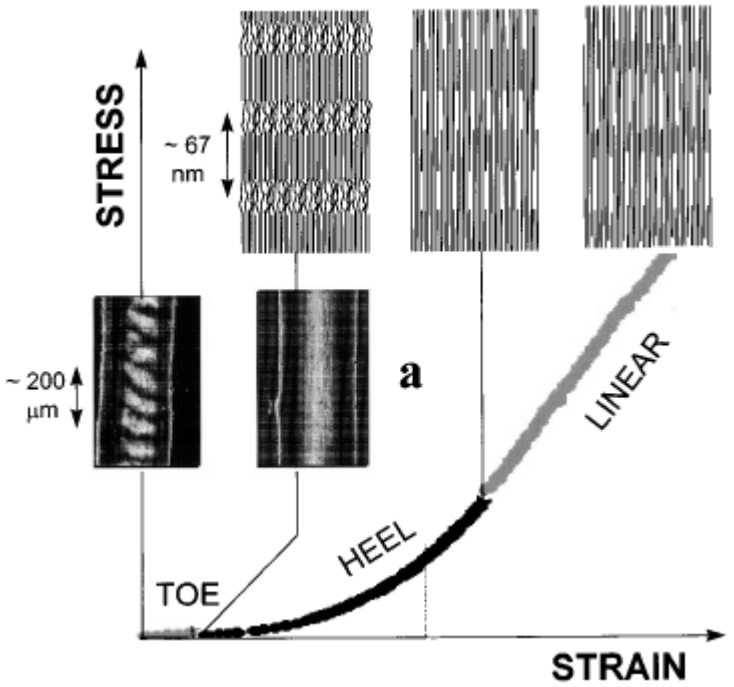

Figure 2.3 The mechanical properties of tendon are largely correlated with the disassociation of bonds within and between collagen fibers. (Image: Fratzl et al. 1997)

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3 Tendon Physiology

3.1 Tendon adaptation to mechanical stimulation

Tendons are composite tissues able to transduce internal and external mechanical signals to effect changes in tissue structure and function. It has been postulated that different structural elements within the cytoskeleton are responsible for detecting different forms of load (Jamney & Weitz, 2004). Gene expression profiles adapt to dynamic chemical and mechanical stimuli to maintain adequate properties for the changing environment (Almekinders et al., 1993; Banes et al., 1995; Archambault et al., 2002; Tsuzaki et al., 2003). Collagen synthesis and tensile modulus have been shown to increase as a result of physiologic loading conditions, while lack of loading resulted in decreased strength of the tendon. (Hannafin et al., 1995) Mechanical overstimulation has been shown to induce the release of cytokines and degradative enzymes (Banes et al., 1995, 1999; Tsuzaki et al., 2003). In contrast, mechanical understimulation of tendon cells can lead to patterns of catabolic pathways, resulting in extracellular (ECM) matrix degradation and loss of material properties, ultimately leading to degenerative diseases (Lavagnino et al., 2003, 2005; Arnoczky et al., 2004).

For years, mechanical loading has been recognized as a vital force in the development and maintenance of cells and tissues, including proliferation, differentiation, gene expression, and matrix metabolism (Banes et al., 1995).

Scientists have prepared a 'tensegrity model' as a paradigm of mechanosensitivity. In this model, it is hypothesized that cells and their nuclei respond immediately to mechanical forces via cell surface (integrin) receptors that are physically connect the cytoskeleton to the ECM (Ingber, 1993; Wang et al., 2001). As an elastic medium, the cell can be deformed by the surrounding, more rigid ECM when load is applied. Also, when forces are generated within the cell, the matrix is able to resist, resulting in changes in cell shape and function. Deformation of any part of the cell produces tensile changes within the cytoskeleton, alerting the cell nucleus of a mechanical change (Wang et al., 1994, 2004; Banes et al., 1995). Deformation of the cell membrane can also open or close ion channels, which control the influx of second messenger molecules such as Ca^{2+} and IP_3 (Banes et al., 1995). In turn, these messengers can activate a gamut of cellular processes, including DNA synthesis, mitosis, cell differentiation, and gene expression (Sachs, 1988; Banes et al., 1995).

The integrin receptors which connect cells to their matrix do so through groups of actin-associated proteins. These integrin receptors cluster together, forming focal adhesions which are responsible for anchoring cells to their matrix. These points of attachment are thought to be the main cell-matrix force transmitters. In this way, the matrix is able to modulate cellular function, based on the ECM's ability to balance the imposed stresses during loading conditions (Ingber, 2003).

Cells also have the ability to exert forces on the substrates (Sawhney & Howard, 2002; Qi et al., 2007). Cells cultured on a flexible membrane are capable of contracting the material, as are cells cultured within a 3D collagen gel construct (Harris et al., 1980; Garvin et al., 2003; Triantafilopolous et al., 2004; Qi et al., 2007).

Interestingly, as the substrate material becomes stiffer, cells exert more force on their surroundings (Lo et al., 2000). Furthermore, cells align in the direction of principle strain, as is seen in the anatomy of tendon *in vivo* (Eastwood et al., 1998; Jones et al., 2005).

Lavagnino et al., and Arnoczky et al., have shown tenocytes may be calibrated to respond to specific levels of strain (Lavagnino et al., 2003, 2005; Arnoczky et al., 2004). The balance achieved through cytoskeletal reorganization after a stress is applied to cells may be the mechanism through which cells maintain this set-point. Changes in cytoskeletal tension control a subsequent change in expression of anabolic and catabolic genes (Lavagnino & Arnoczky, 2005). However, the apparent ability to return to normal expression profiles suggests tenocytes are able to recalibrate their set-point to external stresses (Lavagnino & Arnoczky, 2005). For altered response conditions, however, this tensional homeostasis may be inaccurate for the presented mechanical environment.

The importance of cell-matrix interactions has been the focus of many scientists. Archambault proposed an algorithm of the pathogenesis of tendinopathy whereby tendon cells are not able to maintain the extracellular matrix of the tendon (Archambault et al., 1995). In this mismatch scenario, degeneration of the ECM, and ultimate weakening of the tissue result in increased risk of damage during normal loading conditions. Thus, it is important to understand the cellular machinery responsible for load responses.

3.2 Tendon Pathology

The Achilles tendon is the strongest and largest tendon in the human body. Despite its impressive biomechanical behavior, the Achilles tendon is a site of common athletic injury. Repetitive motion injuries, like those that accumulate with many sports, occur when micro-damage accrue at a rate greater than the body can repair and may lead to a catastrophic failure of the tissue (Jarvinen et al., 1997). Tendon pathology, pain, degeneration, and rupture are the major causes of sports-related injuries, yet remain an enigma in modern medicine.

Tendons may be affected by a variety of different pathological conditions. They vary considerably with multiple factors such as increased age, reduced vascular perfusion, anatomic variation, occupation, and activity level. (Leadbetter, 1992; Kannus, 1997; Riley, 2004) Tendonosis is the term conventionally used for chronic painful tendon associated with inflammation (Ernest et al., 1991; Den Hartog et al., 2003). Spontaneous tendon rupture is a catastrophic failure event marking the complete failure of the tissue without any preceding clinical symptoms. (Kannus, 1991) The impressive biomechanical properties of tendon, however, typically prevent a complete failure from occurring without some degree of matrix degeneration. (Gibson, 1998) Thus, it is believed that a series of sub-failure fractures occur with overuse. This is the case in repetitive motion injuries, where repeated low-level loading of the muscle-tendon unit (Kannus, 1997) Tendinopathy is a major problem associated with overload and overuse resulting from sports and physical activity in active people over 25 years of age (de Mos et al., 2007).

Tendon rupture results in bleeding, clotting, and release of PDGF, TGF-β, ATP, and ADP from platelets, release of hormones such as epinephrine and

norepinephrine from blood vessels and/or nerves, activation of IGF-1 from plasma and tendon matrix, and TGF-β from matrix at the wound site (Beigi et al.,1999; Almekinders, 1993; Ackermann et al., 2003; Tsuzaki et al., 2003). One of the most rapid and typical cellular responses to mechanical load is the increase in intracellular calcium, mediated by the release of extracellular nucleotides.

After tendon injury, inflammation can occur with influx of white cells, expression of cytokines and metalloproteinases, and swelling (Riley, 2004). However, most experts in the field believe that tendinopathies do not involve a classic inflammatory pathway, but rather involve a local molecular inflammation caused by resident cells that express MMPs, COX2, and make PGE_2 (Tsuzaki et al., 2003, Riley et al., 2003, 2004).

The tenocyte is thought to play a pivotal role in the pathology of tendinopathy. Tenocytes secrete and degrade the extracellular matrix, a process that is normally well-regulated and essential for the maintenance of the structural properties of the tissue. However, an imbalance of the catabolic and anabolic activities has been implicated in other degenerative tissues such as osteoarthritis (Clark & Parker, 2003), which suggests reduced matrix synthesis or increased matrix degradation may be targets for future drug therapy of tendinopathy.

The molecular portrait of a pathologic tendon is characterized by glycoaminoglycan (GAG) accumulation, calcification, and lipid accumulation (Riley, 2004). Also, expression and activation of enzymes involved in matrix degradation have been most closely linked to tendon pathology (Riley, 2004; Tsuzaki et al., 2003). Matrix metalloproteinases 1, 2 and 3 (collagenase, gelatinase and

stromelysin) have been key enzymes found in pathologic tendon (Alfredson et al., 2003; Riley, 2004). MMP1 is the most powerful enzyme based on amount and activity, but it is the least highly expressed. MMP2 degrades denatured collagen and is therefore only active once collagen triple helices have been initially degraded by MMP1 or another enzyme. MMP3 degrades proteoglycans, but also activates MMP1. MMP3 was least associated with tendons isolated from painful Achilles tendons or supraspinatus tendons (Riley, 2004).

3.3 Tendon healing

Tendons are inherently strong structures able to withstand considerable force. However, when this tissue fails it can affect the musculoskeletal system in debilitating ways. Current clinical treatments, however, are largely theory based. Thus, a better understanding of the mechanical and biological events that occur in tendon injury will aid in the development of both treatment and preventative strategies for specific tendon disorders.

Tendons possess intrinsic and extrinsic healing abilities. However, complete regeneration of the tendon is never achieved as a lack of cells and reduced collagen fiber diameter alter the mechanical integrity of the repair tissue. Lack of vascularization is the main impediment preventing rapid healing in tendon. Animal models consistently demonstrate enhancement of mechanical properties in response to controlled mobilization and conversely impaired by immobilization (Kvist, 1994). Controlled motion therapy and static stretching demonstrated an increase in collagen mass and fibril size, vascularization, range of motion, and

strength (Gelberman et al., 1983; Jozsa & Kannus, 1997). However, this loading regime must be properly timed in the healing phase.

In the event of a complete rupture, an inflammatory phase lasts one week post-rupture. Days 3-7 involve cells migrating from the peritendinous tissues (Reddy et al., 1999). In the following five weeks, the tendon tissues begin to regenerate with increased tenocyte proliferation and collagen synthesis. Originally randomly oriented, tenocytes play an active role in synthesizing and organizing the newly formed matrix. During this time, the mechanically superior collagen I is produced in preference to type III collagen; this results in an increased strength of the repair. Between weeks four and six, the response transitions to the remodeling phase. This portion of the healing response achieves final stability and also involves the introduction of normal physiological loading conditions.

3.4 Conclusion

Mechanical load induces a wide range of physiological responses, including altered gene expression, cell migration, proliferation, and increases in intracellular calcium. While mechanical forces are necessary for tendon survival, excessive amounts can lead to negative effects on the material and structural properties of the tissue. The response of tenocytes to the changing mechanical environment has significant implications in understanding the pathogenesis of tenodinopathy. Thus, the exact mechanisms of mechanotransduction continue to be a focus for scientists. In the scope of this work, the role of purinoreceptors in mechanotransduction is investigated. This research can potentially lead to controlling load responses in

damaged or deteriorating tendons, as well as broaden the understanding of nucleotides in the load response.

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4 Mechanotransduction in Tendons

4.1 Cell-cell signaling

In all multicellular organisms, survival depends on a complex intercellular communication network that coordinates the growth, differentiation, and metabolism of the multitude of cells in diverse tissues and organs. Cells within small groups often communicate by direct cell-cell communication. This is accomplished through a signal that is received, processed internally, and transduced to other cells. In mechanotransduction events, the initial signal is mechanical, which the cells translate into chemical signals to influence cellular and extracellular components. The ability of tenocytes to respond to load is mediated through activation of several secondary messenger pathways within the cell (Figure 4.1). Activation of any of these pathways leads to mechanically-mediated adaptation, including altered regulation of genes that synthesize and catabolize extracellular matrix proteins, as well as variations in cell proliferation.

Tenocytes respond to mechanical events via multiple redundant G-protein coupled receptor (GCPR) mechanosensory pathways and second messengers, including: stretch-activated ion channels, Ca^{2+} channels, gap junctions, purinoreceptors, integrin interactions with the matrix, and cytoskeleton (Banes et al., 1993,1995, 2001). Calcium is a ubiquitous second messenger used by virtually every cell type and is involved at some stage in a wide variety of cellular processes.

These cellular activities are synchronized through cell-cell signaling events such as intercellular calcium signaling. This mechanism of cellular communication has been studied in a variety of cell types, including tendon (Boitano et al., 1992; Sanderson, 1996; Banes et al., 1994, 1995; Goldberg et al., 2002; Jones et al., 2006).

Calcium transients have been observed in various cells types where they coordinate physiological responses within the respective tissues. Although Ca^{2+} is involved in these responses, it is not required to transduce mechanical stimulation or initiate intercellular communication (Sanderson et al., 1990). Instead, there are multiple mechanisms by which the spread of Ca^{2+} can occur. One relies on the diffusion of IP_3 through gap junctions that physically connect neighboring cells, thus allowing the spread of a calcium wave (Boitano et al., 1992, 1994; Banes et al., 1995; Sanderson, 1996; Wall et al., 2006; Jones et al., 2006). Another means by which intracellular calcium becomes elevated is through a paracrine route that involves the binding of released ATP or other nucleotides to their receptors (Grierson & Meldolesi, 1995; Sauer et al., 2000; Homolya et al., 2000; Schwiebert et al., 2000). This activity was first observed when cells lacking gap junctions were able to induce an intercellular calcium wave, thought to be mediated by extracellular ATP acting on purinoreceptors (Sanderson et al., 1996). An assortment of other molecules including norepinephrine (Wall et al., 2004), nitric oxide (Dedkova et al., 2007), PGE_2 (DeCesare et al., 2006), and other prostaglandins (Krizanova et al., 2007) also activate the calcium signaling cascade to establish cellular cooperation. Moreover, multiple mechanisms may be involved concurrently (Nihei et al., 2003).

Mechanical loading such as laminar shear stress, compression, tension, and osmotic swelling cause the release of ATP in almost every cell type examined to date, including tenocytes (Cotrina et al., 1998; Lazarowski et al., 1997; Tsuzaki et al., 2004; Bowler et al., 2001; Dixon et al., 2003; Weibe et al., 1999). ATP release occurs by transport through the ATP binding cassette proteins, one of which is the cystic fibrosis transmembrane regulator (CFTR), connexin hemichannels, pannexins, and the $P2X_7$ receptor (Abraham et al., 1993; Schwiebert et al., 1999; Beigi et al., 1999; Cotrina et al., 1998; Locovei et al., 2006; Schneider et al., 2006).

4.2 Gap junctions

Gap junctions are intercellular channels that consist of two hemi-channels, termed connexons, localized in the membranes of adjacent cells and allow the passage of hydrophilic mediators and diffusion of second messengers between adjacent cells. Gap junctional communication is essential for proper development and health in mammals, and aberrant connexin expression has been associated with specific disorders (Bergoffen et al., 1993; Lo, 1996; Kelsell et al., 1997; Goldberg et al., 2002; van Steensel, 2004; Laird, 2006; Apps et al., 2007). Tenocytes *in vivo* and *in vitro* have immunohistochemically detectable gap junctions (McNeilly et al., 1996; Wagget et al., 2006; Wall et al., 2007). Gap junctions are created across the intervening extracellular space by the docking of two hemichannels contributed by each adjacent cell (Goodenough, 1996; Kumar & Gilula, 1996). Each hemichannel is an oligomer of six connexin molecules; thus, the complete gap junction represents a dodecameric complex (Yeager et al., 1998; Evans et al., 2000). In this way, gap

junctions serve as a direct intercellular passageway for second messengers (of less than 1kDa) without the use of extracellular space to exchange the messages (Goodenough, 1976; Bruzzone, 2001).

Over 20 members of the “connexin” gene family have been identified thus far differing in impedance, permeability, and pattern of expression (Goodenough et al., 1996; Simon & Goodenough, 1998). Each of theses isoforms is identified with numerical suffixes referring to the molecular weight in kilodaltons (e.g., connexin 43 or Cx43). The most commonly studied gap junction proteins are Cx26, Cx32, and Cx43.

All connexins share the structural motifs of four transmembrane domains, intracellular amino and carboxyl termini, a cytoplasmic loop, and two extracellular loops involved in docking interactions with connexins of touching cells (Goodenough, 1996; Yeager & Nicholson, 1996; Evans et al., 2000,). Close cell-to-cell apposition is necessary for gap junction assembly. Therefore, cell adhesion molecules are candidates that may aid the formation of gap junctions; reports have confirmed that cell adhesion molecules can modulate gap junction formation (Shaw et al., 2007). Cells that lack cadherins showed reduced gap junctional communication, which was then restored when cell adhesion molecules were reintroduced (Musil et al., 1990, Jongen et al., 1991).

The majority of connexins has been shown to be phosphoproteins and thus may be modified post-translationally by phosphorylation, primarily on serine amino acids (Musil et al., 1990; Berthoud et al., 1997). Phosphorylation has been implicated in the regulation of a broad variety of connexin processes, such as the

trafficking, assembly/disassembly, degradation, as well as the gating of gap junction channels (see Lampe & Lau, 2000 for review). Connexins are targeted by numerous protein kinases, of which some have been identified: protein kinase C, mitogen-activated protein kinase, and the v-Src tyrosine protein kinase (Warn-Cramer et al., 1996; Saez et al., 1997; Lampe et al., 2000). Data available thus far indicate an expanding role for phosphorylation in the regulation of connexin processing, including intercellular calcium signaling (Musil et al., 1990). In response to a mechanical event, elevated calcium levels activate the release of IP_3 (Berridge et al., 1984, Sneyd et al., 1995). IP_3 diffuses to adjacent cells through gap junctions, causing the releases of Ca^{2+} from the internal stores of cells adjacent to the stimulated cell (an intercellular calcium wave) (Boitano et al., 1992).

Gap junction channels are dynamic structures that open and close in response to changes in the cell. The half-life for Cx43 in cultured cells has been reported to be 1-3 hours (Musil et al., 1990; Crow et al., 1990; Laird et al., 1991), indicating that regulation of gap junction turnover may be an important control factor in intercellular communication. In the case of a damaged cell, a high concentration of Ca^{2+} ions enters the cell, causing the gap junction channels to immediately close (Bennett & Verselis, 1993; Saez et al., 1993). This serves a protective mechanism in which an individual cell is isolated when its calcium level becomes too high as a result of damage or stress (Saez, 2003).

Gap junction expression is closely associated with mechanical forces. Wall et al. found that Cx43 expression is regulated by strain, and may serve as an anabolic signal to increase intercellular signaling in response to loading (Wall et al., 2004).

Moreover, gap junctions have been shown to modulate load induced DNA and collagen synthesis (Banes et al., 1999). Therefore, improved cell-cell communication may result in increased organization in response to load post-injury and throughout the healing process.

4.3 Purinoreceptors

Initially gap junctions were thought to modulate calcium wave propagation induced by mechanical stimuli. However, cells lacking connections necessary for gap junction transport were able to participate in a collaborative response (Sanderson et al., 1990). Later it was discovered that calcium signaling could occur by an alternate route via the release of extracellular nucleotides, such as ATP. In addition to its intracellular roles, ATP acts as an extracellular signaling molecule via a rich array of receptors, which have been cloned and characterized (Burnstock, 2007). ATP and other nucleotides activate purinoceptors and induce a release of calcium from intracellular stores through an IP_3-dependent mechanism (Dubyak & El-Moatissim, 1993; Hansen et al., 1993). Moreover, ATP has been shown to mediate a diverse array of biological functions, including cellular proliferation, ion transport regulation, DNA synthesis, and inflammation (Roman et al., 1999; Schafer et al., 2003).

ATP was first recognized in 1929, and continued to grow in importance for its intracellular roles in many biochemical processes (Drury & Szent-Gyrogyi, 1929). However, it was difficult for scientists to recognize that such a simple and ubiquitous compound could be used as an extracellular messenger. Finally, in 1972 Burnstock

claimed ATP was responsible for neurotransmission in the gut, and the potent actions of extracellular ATP on many different cell types also implicated membrane receptors (Burnstock, 1972). These receptors were entitled purinoreceptors which encompassed two classes termed P1-purinoceptors, for which adenosine is the principal ligand, and P2-purinoceptors, recognizing both ATP and ADP (Burnstock, 1972). Furthermore, since their cloning in 1993 purinoceptors are classified into two groups: a.) metabotropic or G protein coupled, characterized by the P2Y class; or b.) ionotropic, with ligand-gated ion channels typical of P2X receptors (Abbracchio & Burnstock 1994, Communi et al., 2000; Burnstock, 2007). Pharmacological profiles, based on agonist potency order, transduction mechanisms, and molecular structure have been established for seven mammalian P2X receptors ($P2X_{1-7}$), and eight distinct subtypes thus far: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13,}$ and $P2Y_{14}$ (Ralevic & Burnstock, 1998; Boarder & Hourani, 1998; Wolff et al., 2005; Burnstock, 2007). Both P2X and P2Y have been implicated in intercellular Ca^{2+} signaling in the presence of exogenous ATP (Hwang et al., 1996; McCoy et al., 1999). Thus, isolating the receptor(s) responsible for the rise in calcium can be difficult.

The $P2Y_1$ receptor family, comprising $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors, activates heterotrimeric G proteins of the G_q family. These subunits are responsible for activating PLC, thus promoting IP_3-dependent signaling (Harden, 1997). The $P2Y_{11}$ receptor also activates G_s and therefore stimulates adenylyl cyclase activity (Communi et al., 1997). The $P2Y_{12}$ family, containing $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, activates $Gi_{/o}$ and inhibits the activity of adenylyl cyclase (Chambers et al., 2000; Zhang et al., 2002).

The effect of secreted ATP is modulated by ecto-NTPases which are expressed by tenocytes and appear to act principally at the cell surface in tendon (Tsuzaki et al., 2004). ATP can be degraded into its metabolites by a cascade of enzymes: ecto-ATPase, ecto-apyrase, ecto-nucleotide pyrophosphatases, and ecto-5'-nucleotidases (ecto-NTPases) (Czajkowski & Barańska, 2002). Grobben et al. (1999) found the principally acting enzyme to be ecto-nucleotide pyro-phosphatase which hydrolyzes ATP into AMP and PP_i. Non-metabolyzable analogs have been an important tool in understanding the effects of a single nucleotide that lacks susceptibility to enzymatic breakdown.

The most important activities of P2Y receptors are ion transport and responses to stress (Burnstock & Williams, 2000). The first indication that P2Y receptors were associated with ion channels was the observation that ATP promoted a Ca^{2+}-activated Cl^- currents when added to the apical surface of epithelial cells (Yen et al., 1997). Nucleotides such as ATP can be used therapeutically to treat defective cAMP-mediated Cl^- channel conductance in cystic fibrosis by acting at $P2Y_2$ receptors (Parr et al., 1994; Knowles et al., 1991; Merten et al., 1993; Olivier et al., 1996). Purinoreceptors mediate the activation of β-unit of PLC which converts phosphatidylinositol (4,5)-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG) (Boarder & Hourani, 1998). IP_3 diffuses through the cytosol and targets the IP_3 receptors on the endoplasmic reticulum, causing release of internal Ca^{2+} stores (Berridge, 1993).

Pannexins are a new group of proteins that share homology with innexins, invertebrate gap junction proteins (Panchin et al., 2000; Panchin, 2005). Pannexin 1

and 2 have been shown to assemble into gap junctions sensitive to gap junction blockers in Xenopus oocytes (Bruzzone et al., 2003, 2005). However, this result has not been recorded in vivo. Additionally, mechanical load and intracellular calcium appear to modulate pannexin1 channels, and have also been implicated in ATP release (Bao et al., 2004; Lovovei et al., 2006), and therefore may play an important role in mechanotransduction.

A variety of physiological or pathological conditions results in the release of nucleotides into the bloodstream (Ralevic & Burnstock, 1998; Burnstock & Williams, 2000). Purinergic signaling is an essential part of cell proliferation, differentiation, and death (Abbracchio & Burnstock, 1998). Moreover, exocrine and endocrine secretion, immune responses, inflammation, pain, platelet aggregation and endothelial-mediated vasodilatation have all been shown to be modulated by P2Y and P2X receptors (Burnstock & Knight, 2004). There are many ways through which a simple compound can generate such an acute response; one of these mechanisms is through calcium signaling.

4.4 Calcium signaling

Calcium is a ubiquitous second messenger that acts at almost every stage of life. It is a powerful mediator of cellular events, including muscle contraction, fertilization, hormonal secretion, and both programmed and accidental cell death. Its effects transpire within a single cell as a result of intracellular spread of this ion (intracellular singling), as well as the propagation of a signal that mediates coordinated responses among cells (intercellular signaling) (Sanderson et al., 1994).

The importance of extracellular calcium concentration, as well as ion channels that regulate its movement is underscored by their involvement as a signaling mediator inside the cell, as well its role as an exogenous mediator to regulate load responses.

The calcium signaling system is represented by a relatively limited number of highly conserved transporters and channels, which effect Ca^{2+} flux across cell membranes and by numerous Ca^{2+}-sensitive effectors (Sanderson et al., 1994). Molecular cascades responsible for the generation of calcium signals are tightly controlled by Ca^{2+} ions themselves. Genetic factors, which tune the expression of different Ca^{2+}-handling molecules according to adaptation requirements, are other means by which this response is modulated (Sanderson et al., 1994). Ca^{2+} ions determine normal physiological reactions and the development of many pathologic processes.

Calcium channels regulate cytosolic Ca^{2+} concentrations (10-100nM) so that levels within the cell are 10,000 times lower than the extracellular environment. Within the cell, Ca^{2+} can be free, bound to proteins, or contained in calcium stores in large concentrations. Most of the Ca^{2+} within cells is stored bound within the endoplasmic reticulum (ER) and the mitochondria. Ca^{2+} pumps located on the surface of the ER maintain intracellular Ca^{2+} concentrations by controlling the ion current across the cell membrane and internal storage holds. In this way, a cell is able to protect against potentially deadly rises in calcium levels.

An inciting event leads to release of stored calcium or the influx of Ca^{2+} ions across the plasma membrane as part of a bi-phasic response (Hassinger et al., 1996; Robb-Gaspers & Thomas, 1995; Sanderson et al., 1988). This is

accomplished through a variety of molecular signals, one of which is IP_3 (Sanderson et al., 1994, 1995). IP_3 is a second messenger produced by PLC action on membrane-bound phophatidylinositol 4,5-bisphosphate (PIP_2) that activates the release of calcium from intracellular stores from the membrane of the ER (Putney & Bird, 1993; Berridge et al., 1998, Venkatachalam et al., 2002). In many cases, Ca^{2+} oscillations utilize intracellular Ca^{2+}, but prolonged oscillations generally require an influx of Ca^{2+} via membrane channels to replenish intracellular Ca^{2+} stores (Sanderson et al., 1994). This is the first part of the bi-phasic rises in Ca^{2+}, followed by the activation of store-operated channels (SOCs) in the cell membrane which allow entry of external Ca^{2+} to replenish the calcium stores (Iino & Endo, 1992; Putney & Bird, 1993; Bird et al., 2004). This second portion of the response is the major source of calcium responsible for the signaling event (Putney, 1986, 1990; Sanderson et al.,1988).

4.5 Conclusions

Extracellular nucleotides act on almost all cells, including tenocytes, through cell surface P2 purinoreceptors to mediate a broad range of physiological events. Cellular actions are modulated by nucleotide-driven intercellular calcium transients. In addition to the IP_3-dependant release of Ca^{2+} from intracellular stores, there is a mechanically induced Ca^{2+} flux across the plasma membrane of the stimulated cells, and the direction of this Ca^{2+} flux is influenced by the extracellular Ca^{2+} concentration. The significance of purinoreceptors in mechanotransduction is an important focus in science. The close association of purinoreceptors and calcium

signaling reinforces the importance of nucleotides and nucleosides in a wide range of physiological mechanisms that if better understood, could lead to the prevention and treatment regimens for pain, inflammation, and possibly even diseases such as tendinopathy.

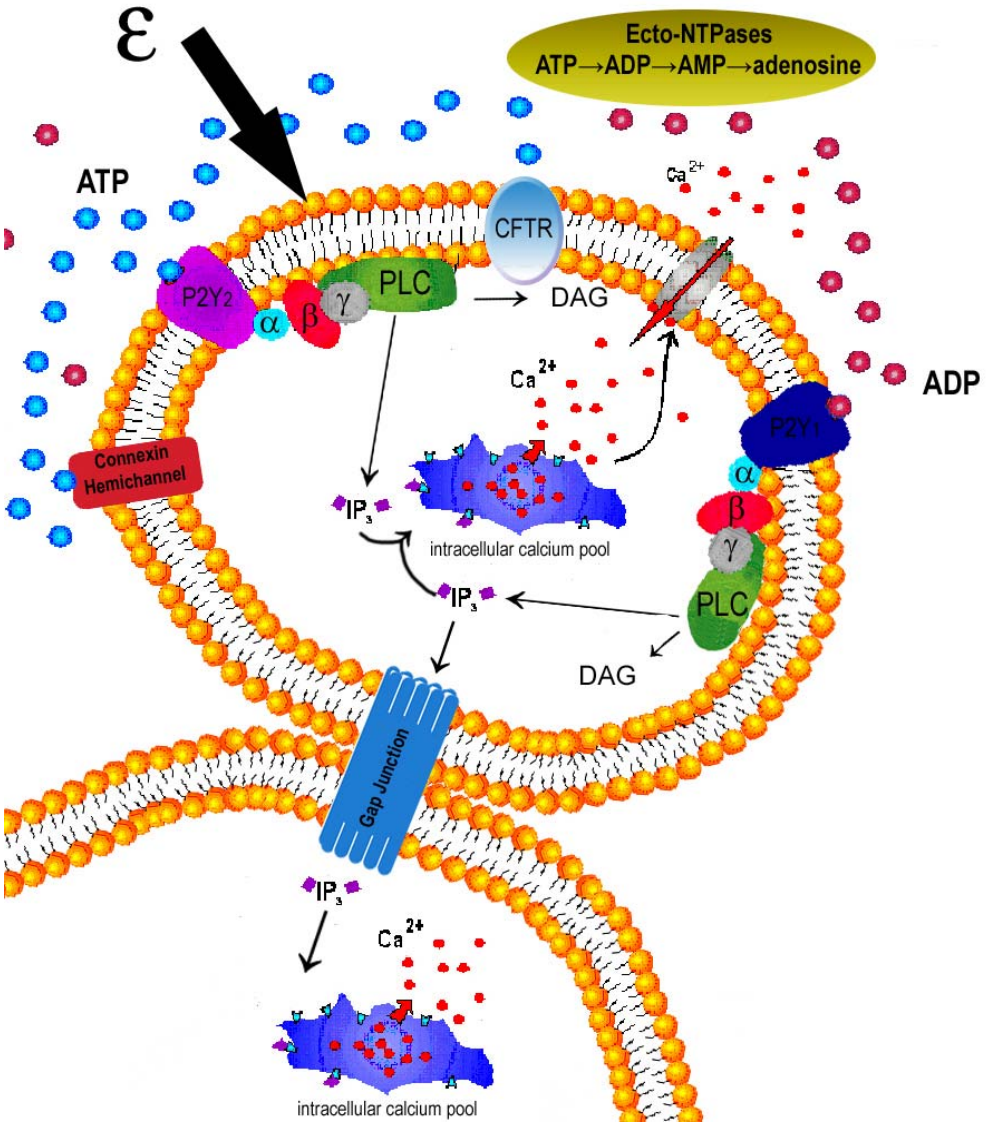

Figure 4.1 Model of purinoreceptor activation pathway in tenocytes in response to load. Application of strain, diffusion of IP_3 through gap junctions, and activation of purinoreceptors through extracellular nucleotides induce a rise in intracellular calcium. This response can be transmitted to neighboring cells through the release of ATP which can stimulate purinoreceptors on neighboring cells, and the diffusion of IP_3 through gap junctions. These GPCRs can work together or antagonistically, in the propagation of an intercellular calcium response. This dissertation focuses on $P2Y_1$ and $P2Y_2$ purinoreceptors activated by ADP and ATP, respectively.

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5 The Structure Function Relationship in $P2Y_1$ and $P2Y_2$ Knockout Mice

5.1 Abstract

A tendon is a tough band of fibrous connective tissue that connects muscle to bone, designed to transmit forces and withstand tension during muscle contraction. The environment provides mechanical input to tissues, which cells detect, respond to, and transmit in order to adapt to the changing demands. One way in which cells, including tenocytes, respond to such conditions is through the release of extracellular nucleotides. Nucleotides such as ATP, ADP, UTP, and UDP activate an increase in intracellular calcium via G-coupled purinoreceptors on the cell surface. This study aims to elucidate the downstream consequences, specifically the mechanical properties, of the genetic deletion of the ADP-sensitive $P2Y_{1,}$ and ATP-sensitive $P2Y_2$ purinoreceptors in tendon. Results of tensile testing indicate $P2Y_1$(-/-) tendons are weaker. Light and electron microscopy provided a means to study the ultrastructural determinants of tendon strength, showing an enlarged collagen fibril diameter. QRT-PCR revealed a downregulation of both type I collagen and decorin – key regulators of connective tissue strength. There exists a clear association between a signaling disparity and the downstream effects that altered intercellular communication has on the structure of tendon.

5.2 Introduction

Tendons join muscle to bone and are responsible for the transmission of forces between the tissues to effect limb movement. Tenocytes that populate these tissues require constant stimulation by mechanical load to preserve the mechanical integrity of the tissue (Lavagnino et al., 2003). Physical stresses such as shear, fluid shear stress, tension, and compression, which occur during daily loading conditions, are responsible for maintaining the unique structural and physiologic characteristics of the tissue. However, it is generally believed that excessive, repetitive loading is the initial event in the development of tendinopathy, leading to degeneration and even rupture (Woo et al., 1982; Archambault et al., 1995; Kannus, 1997; Arnoszky et al., 2007; Banes et al., 2007). To date, there has not been an effective clinical treatment for tendinopathy (Cook, 2007). An emphasis of recent studies is aimed to understand normal tendon metabolism in order to develop therapeutic agents to block matrix degradation (Fenwick et al., 2002; Jones & Riley 2005; Riley, 2005; Burrage et al., 2006).

The ability of a cell to respond to load is the central concept of mechanotransduction and critical to the resulting actions necessary to maintain homeostasis. Tenocytes, the cells that populate tendons, live in a mechanically active environment. The impressive biomechanical properties of tendon rely on a precisely aligned matrix composed primarily of type I collagen bundles interspersed with tenocytes that produce and maintain the matrix. These fibroblasts interpret the mechanical environment and convert that message into a chemical signal.

Tenocytes rely on signaling pathways to achieve a coordinated response to the stimuli through the tissue. The response may involve matrix remodeling to alter the mechanical properties of the tissue, or transcription events to alter the genetic or biochemical state of the cells. Thus, the structural integrity of tendon is cell-dependent. Specifically, the properties of the tissue are conditional on the ability of cells to respond to the mechanical demands of its environment.

A wide range of cellular processes in tendon are induced by the transmission of elevated intracellular calcium concentrations (Berridge et al., 2000). This "calcium wave" is a well-defined response in tendon, which can occur in one or both of the following two ways. The first mechanism propagates the calcium ion release from internal stores by relying on the diffusion of IP_3 through gap junctions, channels that electrically and chemically couple adjoining cells (Boitano et al., 1992). Secondly, bound nucleotides within the cell can be released to act in a paracrine manner on neighboring cells via purinoreceptors on the cell surface.

Nucleotides are a potent yet ubiquitous biochemical signal known to play a variety of roles in connective tissues (Gordjani, 1997; Francke, 1998; Homolya, 2000; Yang, 2000). In particular, ATP and ADP have been implicated as playing a role in tendon mechanotransduction (Tsuzaki et al., 2003, 2005; Elfervig et al., 2003; Banes et al., 2007). Cells detect and respond to these ligands through purinoreceptors, particularly the $P2Y_1$ and $P2Y_2$ subfamily of receptors (Homololya et al.,2000; Sauer et al., 2000). Our lab previously demonstrated tenocytes lacking the $P2Y_1$ ADP receptor have a highly communicated response to both targeted cellular indentation, while those lacking the $P2Y_2$ receptor exhibited a dampened

response to the stimulus. A target cell was deformed, resulting in an increase in intracellular calcium ($[Ca^{2+}]_{ic}$) concentration within the *wt* cells. This reaction is normally transmitted to neighboring cells via gap junctions and/or extracellular signaling through nucleotides. The finding supported a lacking communication among $P2Y_2(-/-)$ tenocytes, while the $P2Y_1(-/-)$ displayed a heightened sensitivity to the stimulation of a single cell. These results indicate a modulatory role of ADP in mechanotransduction.

The negative feedback loop in response to mechanical stimuli is disrupted in $P2Y_1(-/-)$ and $P2Y_2(-/-)$ mouse tail and Achilles tendon cells. Therefore, these knockout mice provide a useful experimental model to understand the roles $P2Y_1$ and $P2Y_2$ purinoreceptors play in maintaining the structural integrity of the tissue. We hypothesized that $P2Y_2(-/-)$ mouse tail tendon tissue would be mechanically inferior to tail tendon tissue from $P2Y_1(-/-)$ mice as a consequence of the cells inability to communicate a response to mechanical poke events. However, the results of tensile tests proved our hypothesis wrong. We also studied histologic and electron microscopy images to understand ultrastructural discrepancies between wt and knockout mouse tendons.

5.3 Materials and methods

5.3.1 Animals and experimental protocol

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. $P2Y_1(-/-)$ and $P2Y_2(-/-)$ mice initially in the B6D2 background (Homolya

et al., 1999), were outbred in the 129SV background (Matos et al., 2005) with gene knockout confirmed by PCR. Age and sex-matched 129SV were used as wild type. Mice were sacrificed by CO_2 asphyxiation according to IACUC protocol.

5.3.2 Light microscopy

A 4mm wide sections were dissected from whole tails isolated from *wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) mice (n=3/group). Samples were fixed in 10X volume of 10% neutral buffered formalin (NBF) for 24 hrs, washed in running water 3X for 3 minutes each, and immersed into 10X volume of Formical-2000 (DeCal, USA). The decalcifying solution was replaced every 24 hours for one week before placed into a container of 70% alcohol. Samples were then taken to the UNC Mouse Histopathology lab, where they were dehydrated in a series of alcohol dilutions and embedded in paraffin.

After cassetting, serial sections of 7μm collected in ribbons, with 8 ribbons per block and 12 sections per ribbon (8 sections per slide with 3 slides per stain) were prepared for staining. Hemotoxylin and eosin (H&E) stains the nuclei of cells blue (with some metachroma) and cytoplasm and muscle pink. Masson's Trichrome creates a staining pattern with blue-black nuclei, red cytoplasm and muscle, and blue collagen musin. A modified CME stain was used to visualize elastin fibers (black), collagen (green), and cytoplasm and muscle (red). Gomori's trichrome was used to evaluate collagen (green) and muscle (red) (nuclei are purple-black).

5.3.3 Transmission electron microscopy

4mm wide section of mouse tail immediately proximal to section taken for light microscopy was isolated from each specimen (n=3/group) (Figure 5.1). Sections were immediately placed in 4% glutaraldehyde for 24 hours and taken to the UNC Dental School Microscopy Center. Tendons were fixed in 2.5% glutaraldehyde/ 2% paraformaldehyde in 0.1M Cacodylate buffer, pH 7.4. Larger samples were cut into strips or cubes about 1 mm wide while in the fixative. Fixation continued for three to16 hours. Samples were transferred to 0.1M cacodylate buffer for rinsing overnight with one change. Samples were stained with 2% OsO4 (in same buffer) for 1 hour and then washed 4x 5 min each with deionized water. Dehydration was carried out using a series of ethanol dilutions (50%, 70%, 95%), for 5 minutes each, followed by two transfers to absolute ethanol for 20 minutes each. Ethanol was then exchanged to propylene oxide with 2 changes (20 minutes each). Samples were placed in a 50:50 mixture of propylene oxide and epoxy resin (Eponate 12 epoxy) (Ted Pella, Inc., Tustin, CA) overnight with stirring, then 100% resin for 8 hours with vacuum. The samples were placed with fresh resin in a flat embedding mold and cured overnight at 65° C. Blocks were sectioned with a Reichart Supernova Ultramicrotome at 90nm. The sections were post stained with uranyl acetate and lead citrate before viewing with a Philips CM12 transmission electron microscope, operating at 100kV accelerating voltage. Images were collected digitally using Gatan's Digital Micrograph. Collagen fibril diameter was digitally measured using Adobe Photoshop.

5.3.4 Materials testing

Tail tendon fascicles from *wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) mice (n=5 mice/group, 10 fascicles/isolation) were gently teased from the distal ends of mouse tails, with care taken not to load tissues. Fascicles were immediately placed in a bath of Phosphate Buffered Saline (PBS) to prevent dehydration. Individual fascicles were placed on a slide and fascicle diameters measured in three locations and averaged, for the purpose of calculating stress (Figure 5.2). Next, fascicles were placed in custom designed grips mounted in an MTS Evolution tensile testing machine (Figure 5.3). A 1000g hermetically sealed load cell (Bose) relayed information to the MTS software continuously as each test was performed. Samples were continually misted with a PBS-filled atomizer during the testing. Loads were calculated to give a normalized strength for tendons (σ (stress)) from the three groups of mice.

Mouse dorsal skin was also tested, as a comparative tissue. An MTS 858 Mini Bionix II equipped with a 250N load cell was used to perform tensile testing of a section of mouse dorsal skin. A dogbone-shaped cutter (2 cm linear portion, 3 cm total length, 0.6cm wide, Figure 5.4) was used to punch skin samples out of the skin once dissected from carcass. Once loaded into grips, specimens were elongated at a rate of 2mm/min until failure. A total of five mice from each group were used for this experiment.

5.3.5 RNA isolation

Tail tendons from *wt*, $P2Y_1$(-/-), and $P2Y_2$ (-/-) mice were placed in a stainless steel mortar pre-chilled with dry ice. Liquid nitrogen was added to the chamber to flash freeze the tissue, and the pestle inserted to powderize the specimen. The

powder was collected into a round bottomed tube and homogenized using a rotor-stator homogenizer for 10 seconds. RNA was isolated using the Qiagen RNeasy Plus Mini kit (Qiagen Inc. Valencia, CA), according to the manufacturer's protocol. RNA yield and integrity was evaluated by spectrophotometry. Reverse transcription was conducted to obtain cDNA to be used in PCR.

5.3.6 Quantitative RT-PCR

Extracted RNA was translated into cDNA using SuperScript III reverse transcriptase per the manufacturer's protocol. Decorin (Forward: GGGCTGGACCATTTGAACAGAGA; Reverse: GAGGTTTGAATGCCTCTGGACTGA) and collagen type I-α1 (Forward: ATTGGTCCCCCTGGTCCTCGA; Reverse: AGTCAGAGTGGCACATCTTGAGGT) primers were used. The cDNA generated was used as template for real time quantitative RT-PCR was performed with a MX3000 QPCR system (Stratagene, USA) using ds-DNA binding fluorophore SYBR™ Green I.

5.3.7 Statistical analysis

Data were analyzed using SigmaStat (SPSS, Chicago, IL) and subjected to one-way ANOVA to determine significance between and among groups. All results were expressed as the mean ± standard deviation, unless otherwise indicated.

5.4 Results

5.4.1 Light microscopy

Gross morphological features were examined using Hemotoxylin and eosin (H&E), Masson's trichrome, a modified CME, and Gomori's trichrome stains. Examination of stained slides shows the fascicle reflects the crimp, or wave, pattern of its constituent collagen fibrils (Figure 5.5). This pattern can display variation between and within each fascicle, and also varies with anatomic location and function of the tendon. The crimp pattern is representative of the elastic capacity of the tissue, and enables tendons to strain in loading conditions, and recoil when the load is removed. Also evident in the pictures are the nuclei of tenocytes. Tenocytes live between collagen fibrils and extend long processes that connect to neighboring cells.

5.4.2 Transmission electron microscopy

TEM reveals the complex ultrastructural organization of tendon, with prominent striations of a periodicity of 67nm (Figure 5.6). This pattern is caused by the overlapping of laterally adjacent collagen superhelicies, which provides an energy release mechanism as they unravel and recoil during loading and unloading, respectively (Evans et al., 1998). Electron microscopy revealed a stark difference in the collagen fibril diameters (Figure 5.7). $P2Y_1$(-/-) tendons displayed a significantly greater average collagen fibril diameter, compared to both wt and $P2Y_2$(-/-) tendons (Figure 5.8) ($p<0.001$). Furthermore, Figure 5.9 shows a histogram depicting the relative distribution of collagen fibril diameters. While *wt* and $P2Y_2$(-/-) tendons show a normal distribution of 50-100nm, the $P2Y_1$(-/-) shows high variation within the samples (50-300nM).

5.4.3 Materials testing

Wt, $P2Y_1(-/-)$, and $P2Y_2(-/-)$ tail tendon fascicles strained in a linear-elastic manner typical of connective tissues (Figure 5.10). Tensile tests revealed a significant decrease in strength in $P2Y_1(-/-)$ tendons (Figure 5.11) ($p<0.001$). The ultimate stress of *wt* and $P2Y_2(-/-)$ tendons were very similar (52.39 +/- 17.66; 52.37 +/- 15.39 MPa), but $P2Y_1(-/-)$ tendons failed at an average of 41.71 +/- 16.66 MPa. Other properties such as diameter and modulus were not significantly different between groups (Figures 5.12, 5.13) Tensile testing of mouse skin followed a similar pattern to tail tendon fascicles; $P2Y_1(-/-)$ samples failed at a lower stress than wt and $P2Y_2(-/-)$ (Figure 5.14). Again, the disparity in ultimate strength is significant, but modulus values did not show a significant difference (Figures 5.14, 5.15).

5.4.4 Quantitative RT-PCR

Gene expression profiles were examined using real time quantitative PCR. Not surprisingly, levels for type I collagen are down nearly ten-fold in $P2Y_1(-/-)$, compared to *wt* and $P2Y_2(-/-)$. Also shown in Figure 5.16, the proteoglycans decorin was found to be downregulated nearly four-fold in the $P2Y_1(-/-)$ tendon tissue.

5.5 Discussion

An important association between purinoreceptors and structural integrity of tendon has been identified in this study. Collagen provides the principle source of mechanical strength in connective tissues and is often implicated in diseases where such structural support is lacking (Prokop & Kivirikko, 1995). It has been shown that both collagen synthesis and tensile modulus increase in response to loading (Banes et al., 1999; Hannafin et al., 1995). Moreover, a lack of applied load results in a decrease in strength of tendon. (Hannafin et al., 1995). Thus, it is clear that changes in loading activity result in a transformation of the matrix, both degradation in the absence of load and synthesis associated with increased loading (Kjaer, 2003).

Maintenance of this homeostasis is dependent upon intercellular communication, including the activation of purinoreceptors. Previous findings revealed a hypercommunicated response to mechanical poking in $P2Y_1$(-/-) tenocytes (Chapter 5). However, tendons from these mice are weaker. Taken together, it is possible that the $P2Y_1$(-/-) cells experience a hyper-response that passes the threshold of normal loading conditions, leading them down a degradative pathway. This would explain the decreased expression of key structural elements in $P2Y_1$(-/-) tendons, which are necessary for the strengthening of a tissue in response to loads. There exists a clear association between a signaling disparity and the downstream effects that altered intercellular communication has on the structure of tendon.

A striking difference was seen in $P2Y_1$ knockout mouse tail tendons, in both strength and structure. Tail tendon fascicles from these knockout mice failed at lower stresses than *wt* and $P2Y_2$(-/-) tendons. In order to better understand the ultrastructural discrepancies that may exist, electron microscopy was used. TEM revealed collagen fibrils that vary greatly in their diameter size distribution in $P2Y_1$(-/-) tendons. Although counter-intuitive, the finding of larger collagen fibrils associated with weaker tendons is supported in the literature (Derwin et al., 2001).

Gene expression analysis revealed decreased expression of type 1 collagen and decorin, two key players in connective tissue strength. Collagen is one of the more important structural proteins in the body being of particular importance in connective tissues by providing their durability. It is the protein thought to dominate the strength of connective tissues, including tendon. The decreased collagen gene expression easily translated into a weaker tissue, evident in $P2Y_1$(-/-) tendon and skin. While collagen is the major constituent that defines the mechanical behavior of tendon, proteoglycans and glycoaminoglycans (GAGs) provide the structural support for collagen fibers and regulate the extracellular assembly of procollagen into mature collagen. Decorin is a proteoglycan that *decora*tes collagen fibrils and is responsible for diameter regulation (Watanabe et al., 2005; Robinson et al., 2005; Reed et al., 2002; Derwin et al., 2001). There is no complete understanding of the mechanisms in transmitting and absorbing tensional forces within the tendon, but is it likely that a flattening of tendon crimps may occur at a first stage of tendon stretching. Increasing stretching, other transmission mechanisms such as an interfibrillar coupling via PG linkages and a molecular gliding within the fibrils structure may be

involved (Franchi et al., 2007). Additionally, decorin knockout mice were associated with the uncontrolled lateral fusion of collagen fibrils, and markedly decreased mechanical strength of skin (Danielson et al., 2006). Thus, it is clear that a decrease in decorin expression could be associated with the increased collagen fibril size of $P2Y_1$(-/-) tendons.

The role of purinoreceptors in collagen formation is unknown. Mouse tail tendons from $P2Y_1$ purinoreceptor-null mice have decreased strength and abnormally large collagen fibril diameters. We propose that the inability to dampen a load response may result in the accumulation of larger, but weaker, collagen fibers.

5.6 Acknowledgements

We would like to thank Dr. Jeffrey Thompson and Dr. Peter Mente for their help and use of tensile testing instruments, and Jeff Piascik for his assistance. Kirk McNaughton for sectioning and staining of light microscopy specimens. The UNC Mouse Histopathology lab was valuable in the paraffin embedding of the light microscopy samples. Dr. Wallace Ambrose of the UNC Dental School was an immense help with the sectioning and staining of TEM samples. Dr. Jie Qi performed the PCR in this study.

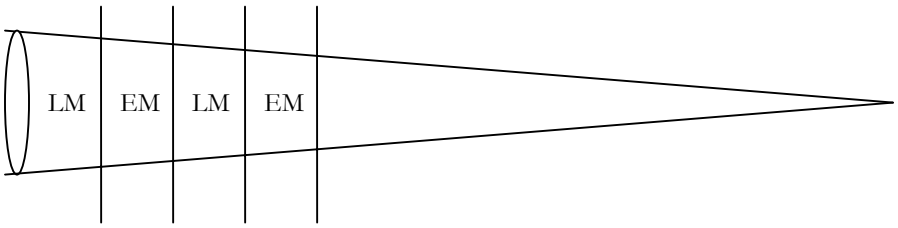

Figure 5.1

<u>Sectioning of the mouse tail followed the outlaid pattern):</u>

- 10 mm for paraffin embedded sections (LM) – cross-section orientation
- 10 mm for electron microscopy (EM) – cross-section orientation
- 10 mm for paraffin embedded sections (LM) – longitudinal section orientation
- 10 mm for electron microscopy (EM) – longitudinal section orientation

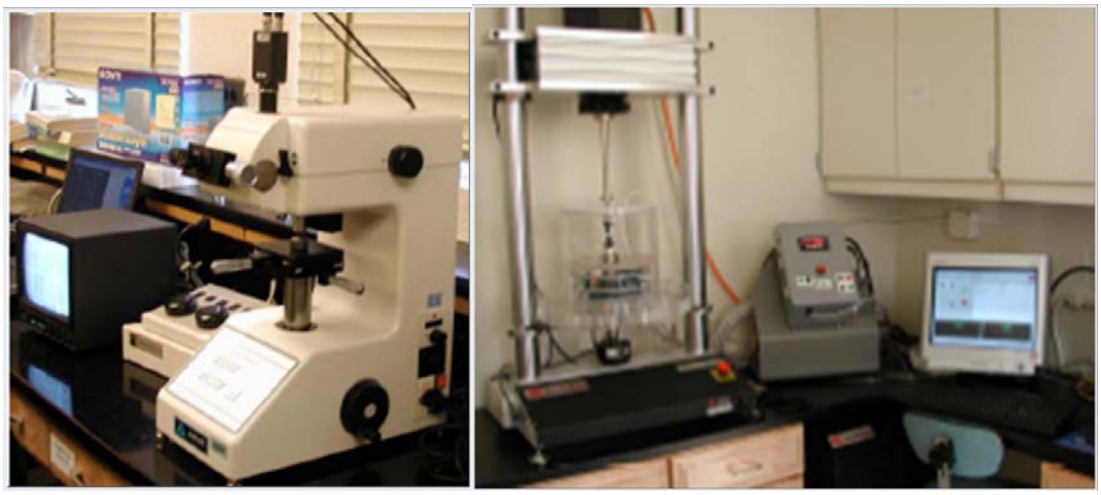

Figure 5.2 a) A hardness tester was used to measure the diameters of each tendon fascicle prior to mechanical testing. b.) Fascicles were tested with an MTS Evolution while continually misted with PBS to prevent dehydration.

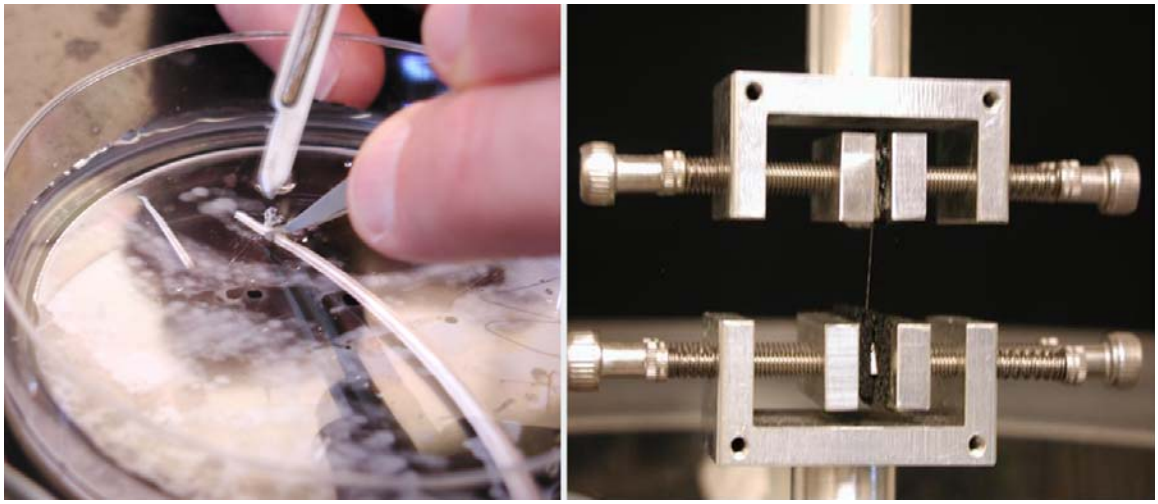

Figure 5.3 a.) Fascicles were gently teased from the distal portion of mouse tail tendons, with care taken not to place strain on the tissues. b.) Individual fascicles were mounted in custom grips and tested until failure.

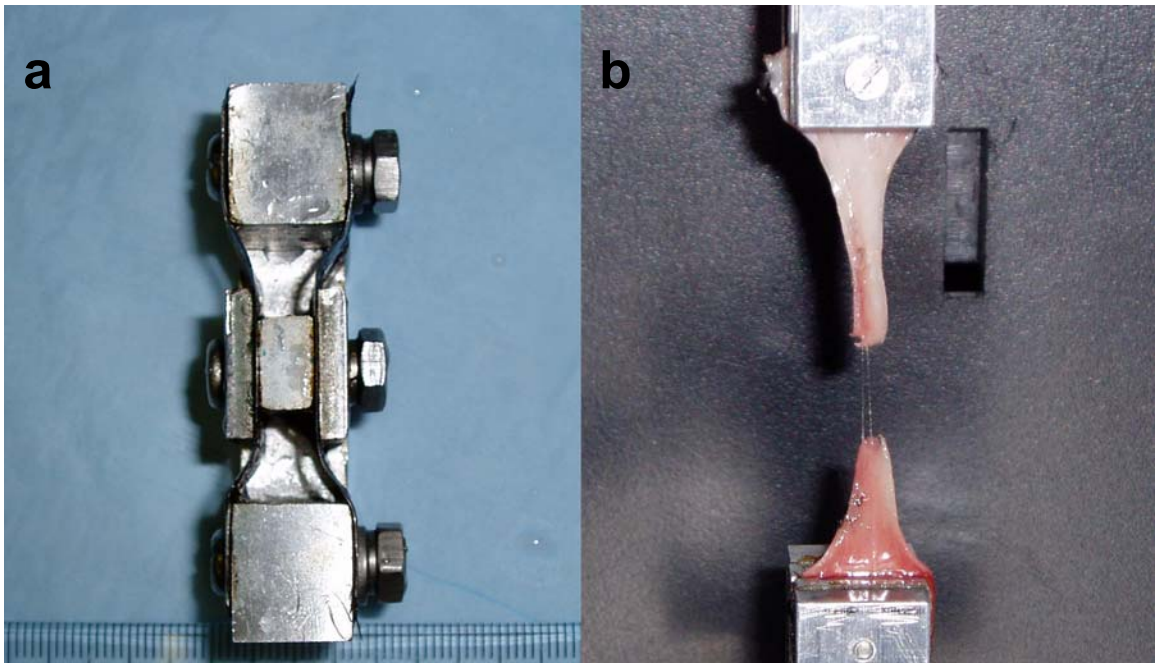

Figure 5.4 a) Dorsal skin specimens from mice were cut using this dogbone-shaped cutter to prevent micro-tears that would lead to premature rupture. b) Complete failure in the center of the samples was common using this technique.

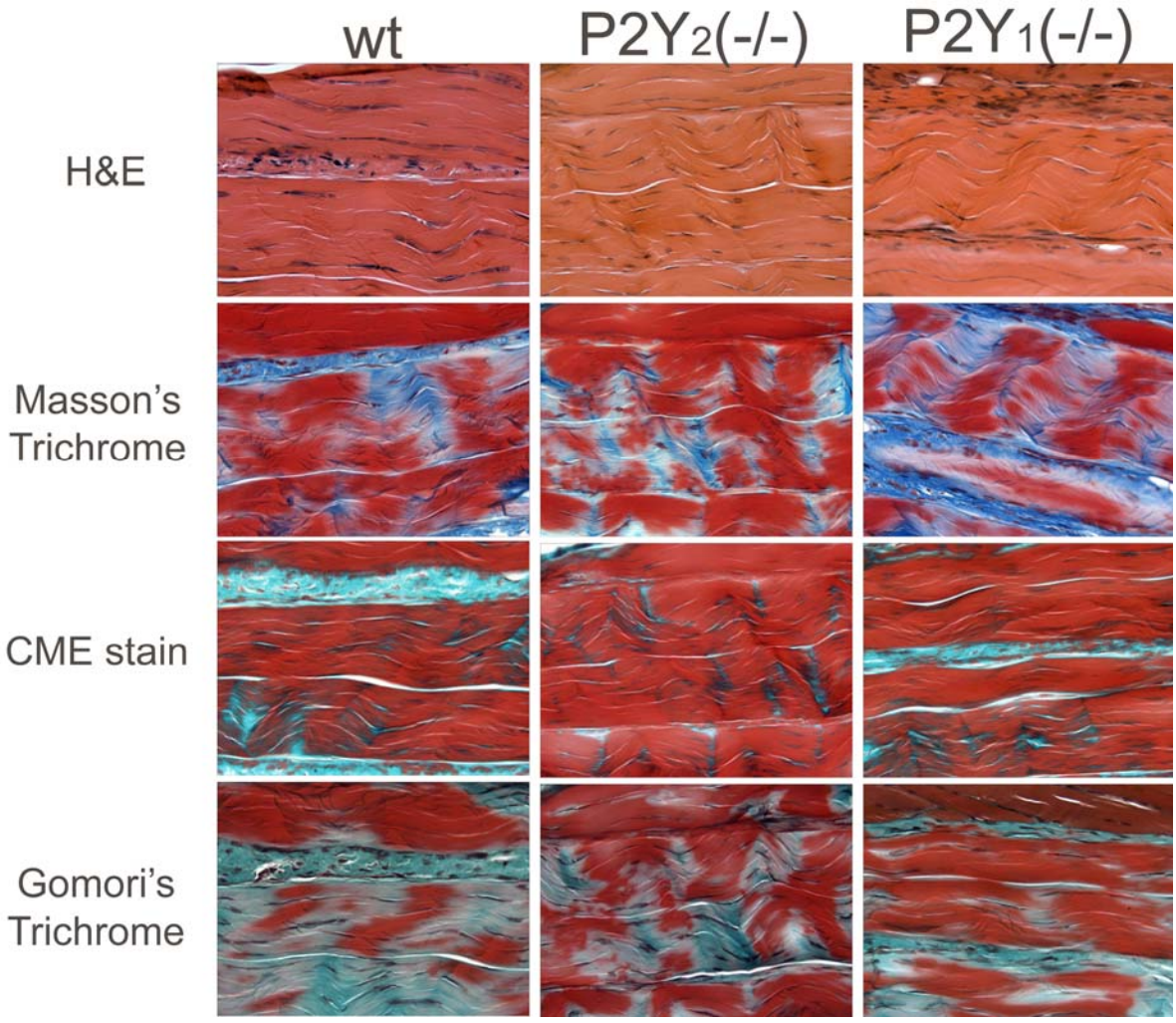

Figure 5.5 Tail tendon sections were stained to examine the cellular and ECM content of the tissues. a) Hemotoxylin and eosin (H&E) stains the nuclei of cells blue (with some metachroma) and cytoplasm and muscle pink. b) Masson's Trichrome creates a staining pattern with blue-black nuclei, red cytoplasm and muscle, and blue collagen musin. c)A modified CME stain was used to visualize elastin fibers (black), collagen (green), and cytoplasm and muscle (red). d) Gomori's trichrome was used to evaluate collagen (green) and muscle (red) (nuclei are purple-black). Periodic undulations are seen in each of the figures above, representing the crimp responsible for energy storage and dissipation in loading and unloading events. Also visible are nuclei of cells interspersed between collagen fibers. Gap junctions at the ends of long cellular processes connect these intermittent cells so that intercellular signaling is possible.

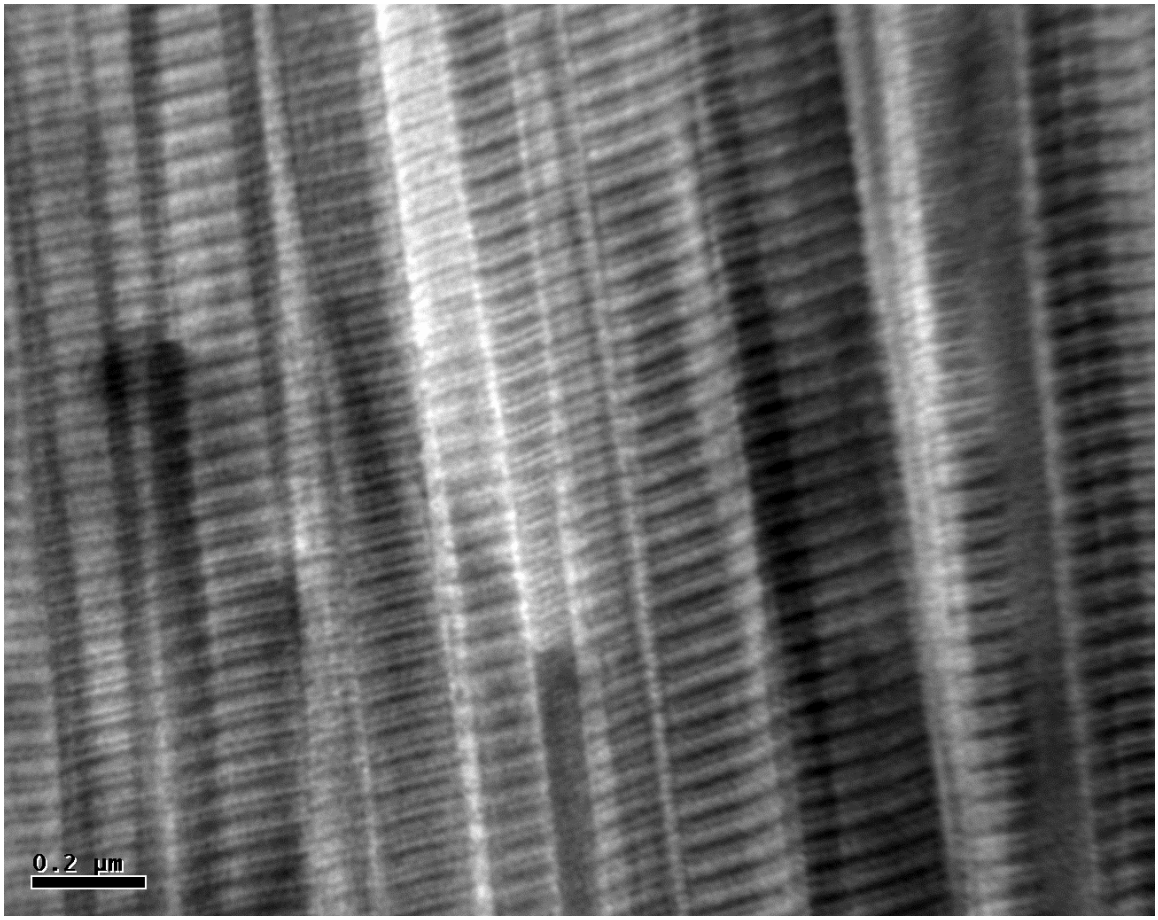

Figure 5.6 The banding pattern seen in this image is caused by the overlapping of laterally adjacent collagen superhelicies. This meticulous design was discernible in *wt*, $P2Y_2$(-/-) and $P2Y_1$(-/-) tendon samples. Scale bar =0.2µm

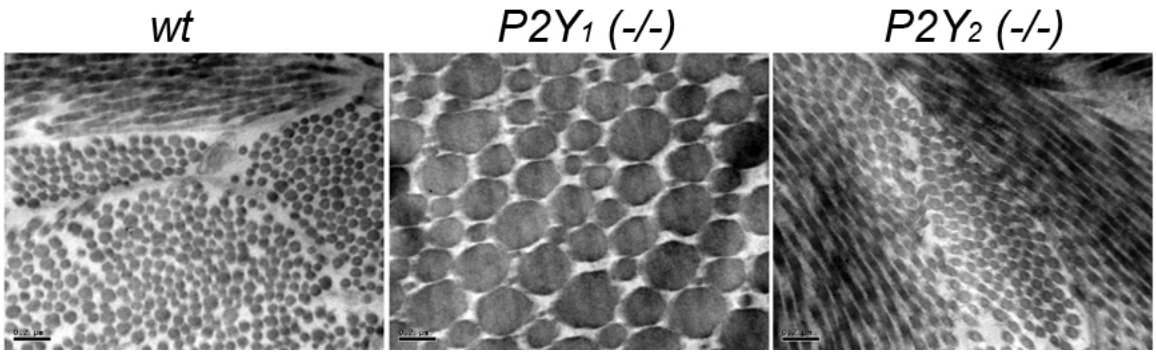

Figure 5.7 Electron microscopy of collagen fibrils (40,000X) from *wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) mouse tail tendons. The $P2Y_1$(-/-) showed a marked increase in fibril diameter distribution and overall size. Scale bar = 0.2μm.

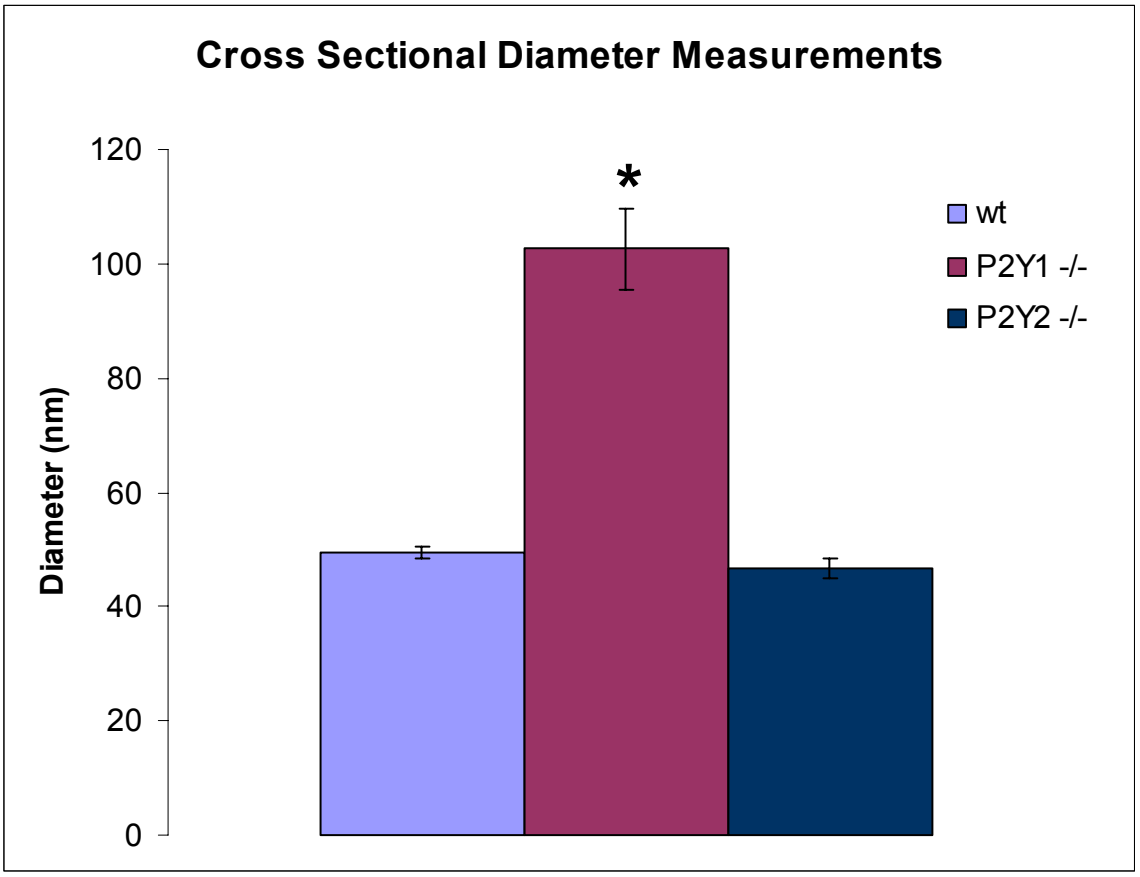

Figure 5.8 Cross sectional diameters were measured for collagen fibrils, as seen in TEM images. Tendons from $P2Y_1(-/-)$ mice showed a dramatically increased value, compared to both *wt* and $P2Y_2(-/-)$. (*=$p<0.001$)

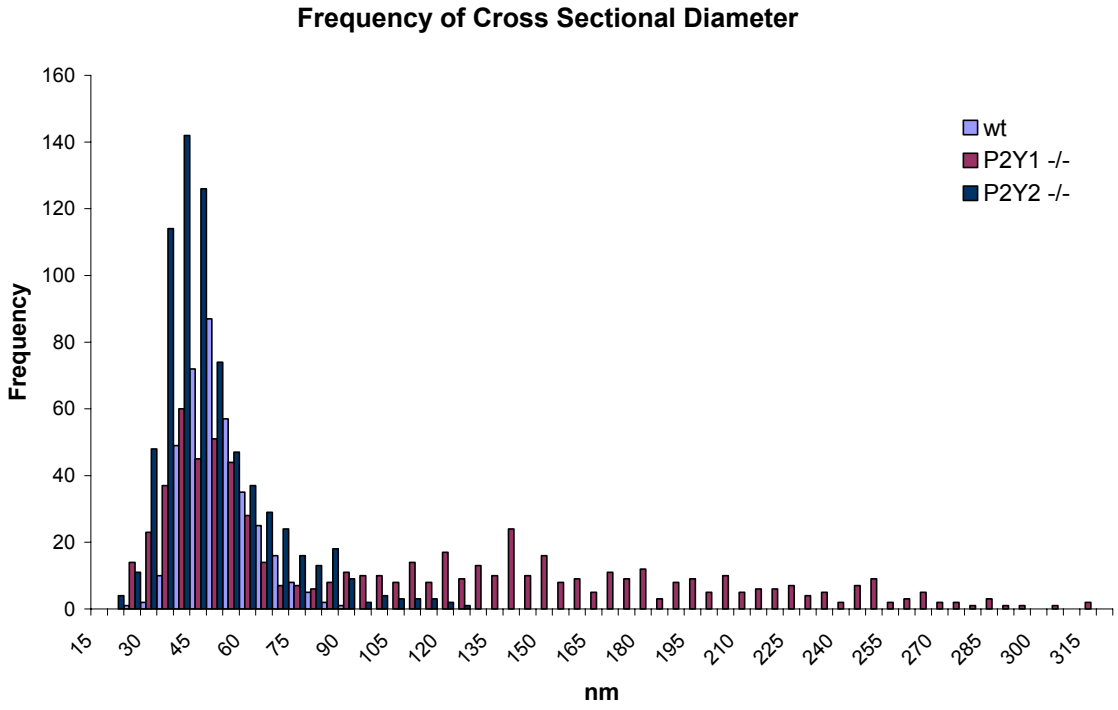

Figure 5.9 In addition to a larger average collagen fibril diameter, this histogram demonstrates the high variability of measurement frequencies of $P2Y_1(-/-)$ tail tendon collagen fibrils, whereas the *wt* and $P2Y_2(-/-)$ are centered around 50nM.

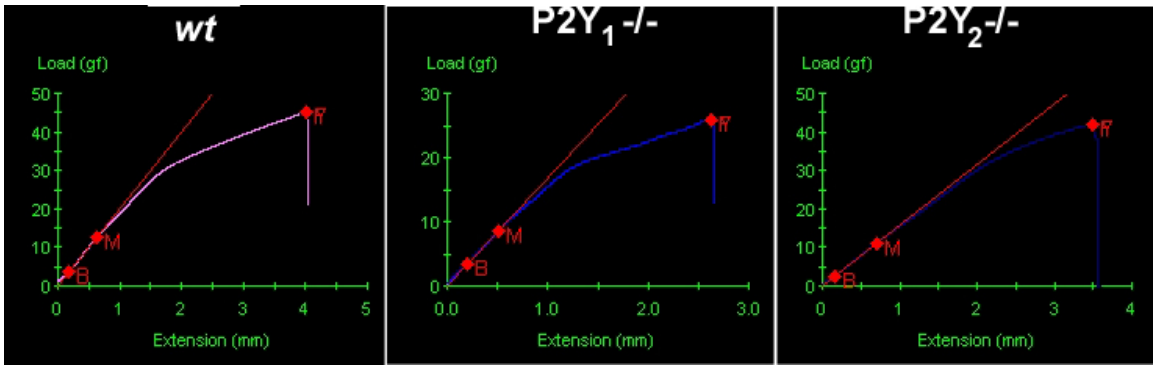

Figure 5.10 Stress-strain graphs for wt, $P2Y_1$(-/-) and $P2Y_2$(-/-) tail tendon fascicles depicted a linear-elastic curve typical of connective tissues. Variation in the shapes of the curves was not evident in the toe or plastic regions, but tendons from $P2Y_1$(-/-) had lower ultimate failure stresses.

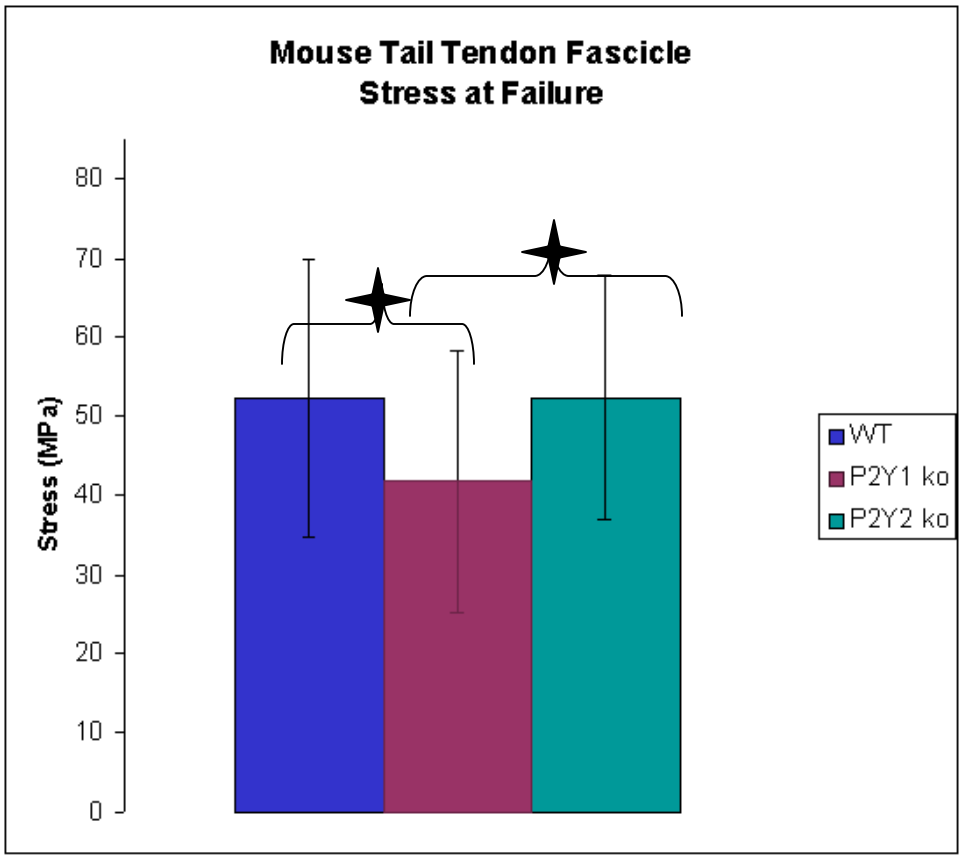

Figure 5.11 Average ultimate stress of tail tendon fascicles. A significant difference was evident in $P2Y_1(-/-)$ tendons compared to both *wt* and $P2Y_2(-/-)$ tendons. ($p<0.001$) There was no significant difference between $P2Y_2(-/-)$ and *wt*.

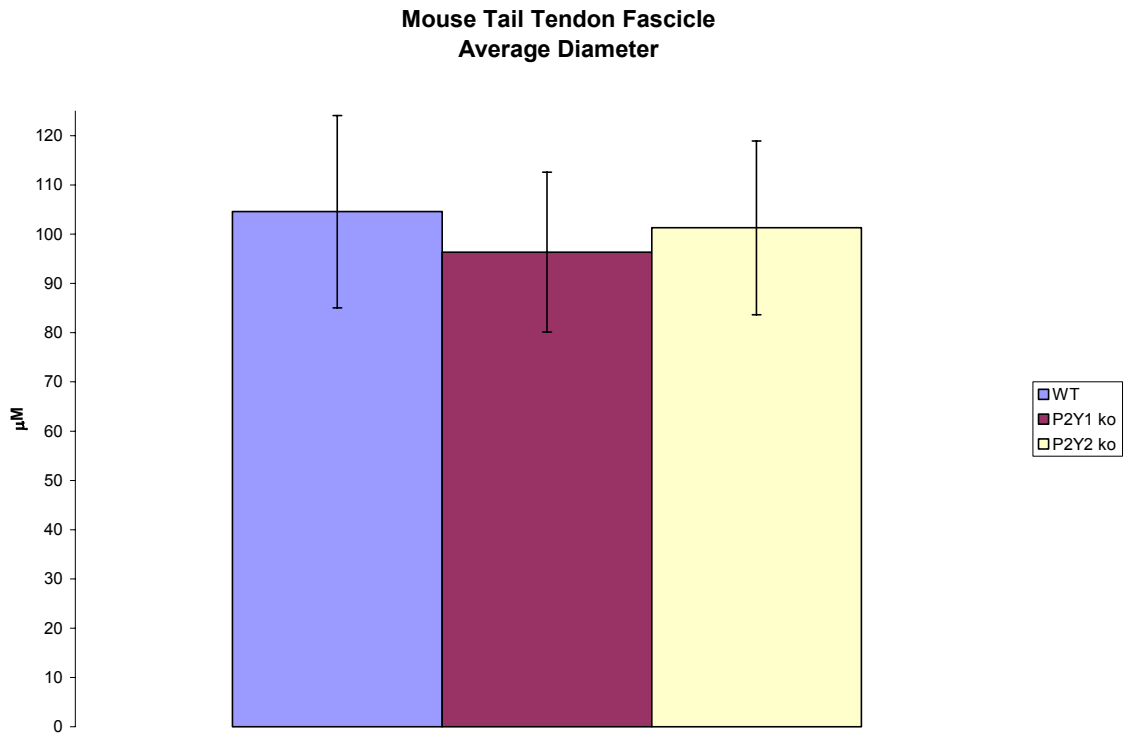

Figure 5.12: Fascicle diameters were measured prior to tensile testing in order to normalize failure loads to stresses. The average diameters were not significant between groups. ($p<0.05$)

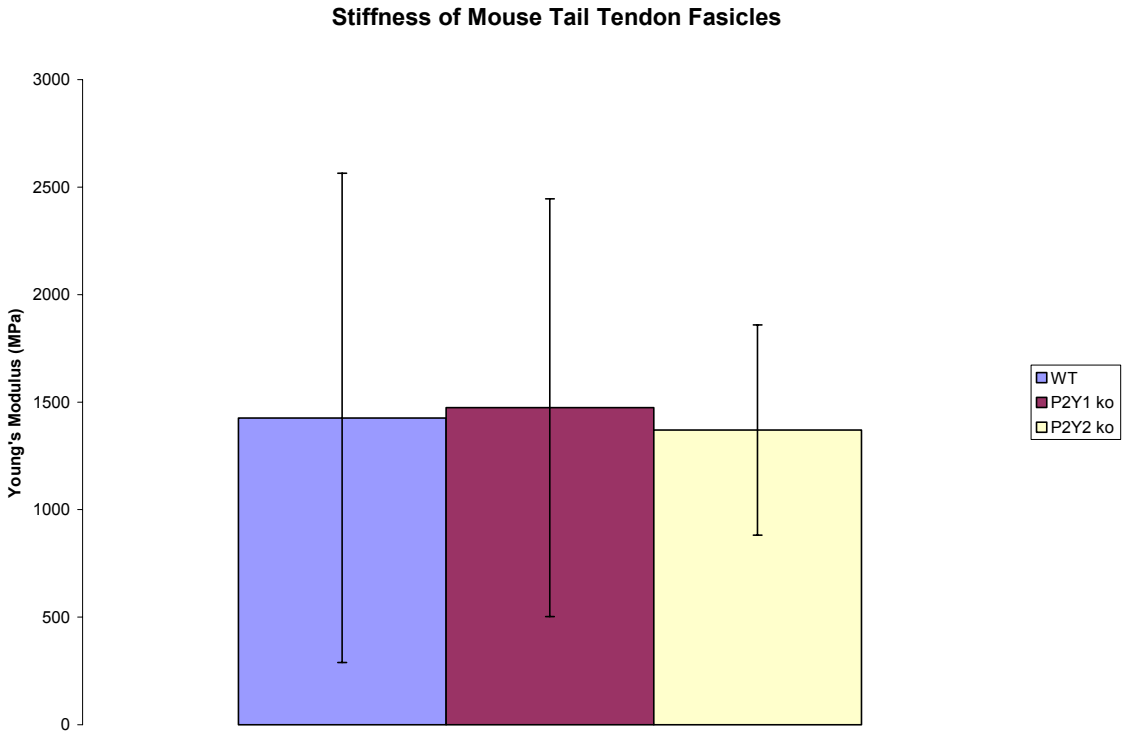

Figure 5.13: Elastic moduli of mouse tail tendon fascicles exhibited great deviation within each group. The calculated stiffnesses of tendons were not significantly different from each other. ($p<0.05$)

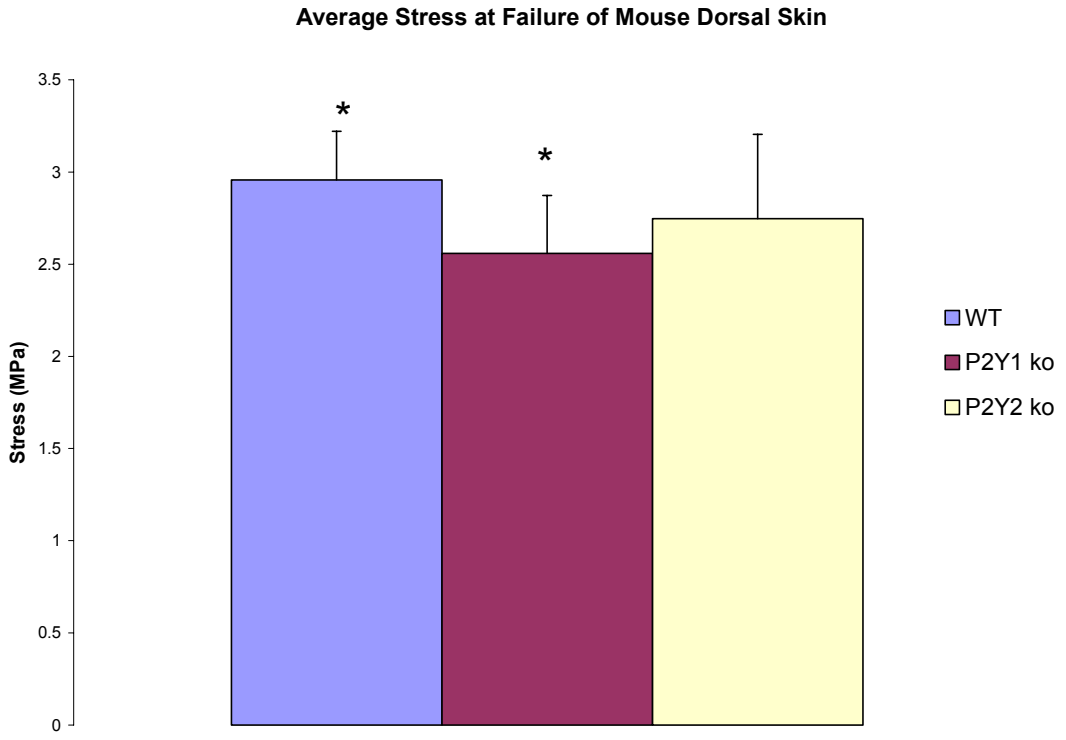

Figure 5.14: Dorsal skin from *wt*, $P2Y_1(-/-)$, and $P2Y_2(-/-)$ mice was tensile tested to explore the mechanical behavior of another type of tissue. As with tail tendon fascicles, $P2Y_1(-/-)$ skin was also weaker. ($p<0.001$) This indicates the structural inferiority is not isolated to tail tendons alone, but may be a phenotype of many tissues.

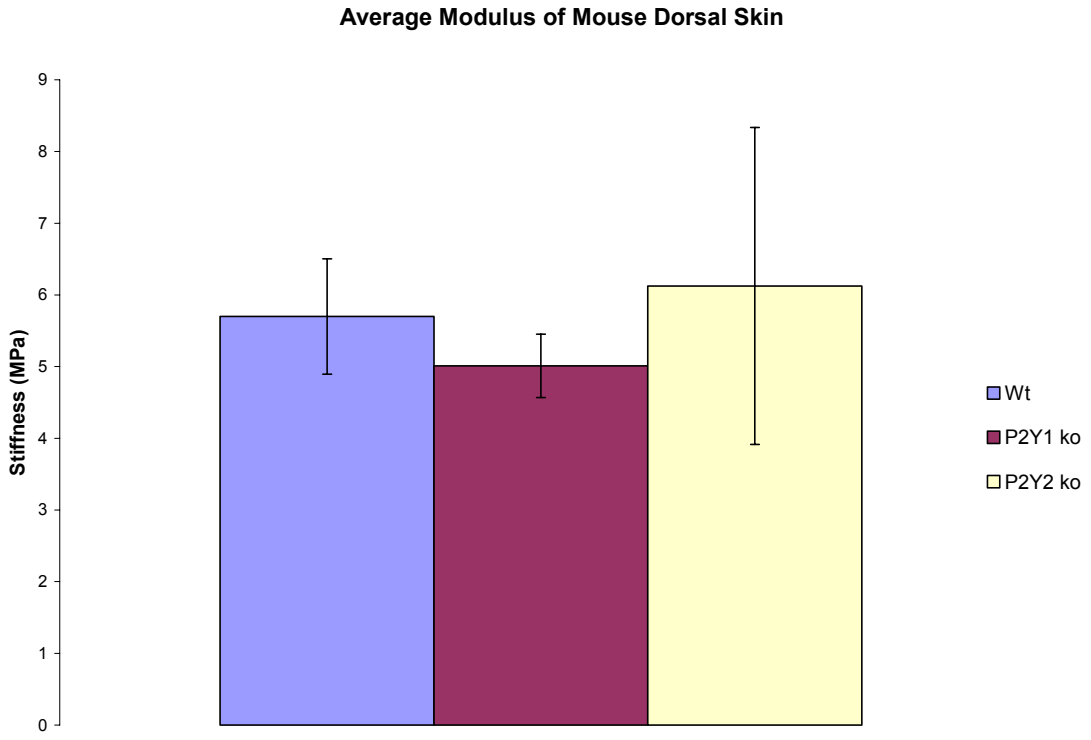

Figure 5.15: The average moduli of mouse skin samples were calculated. No significant difference was seen between groups of mice. ($p<0.05$)

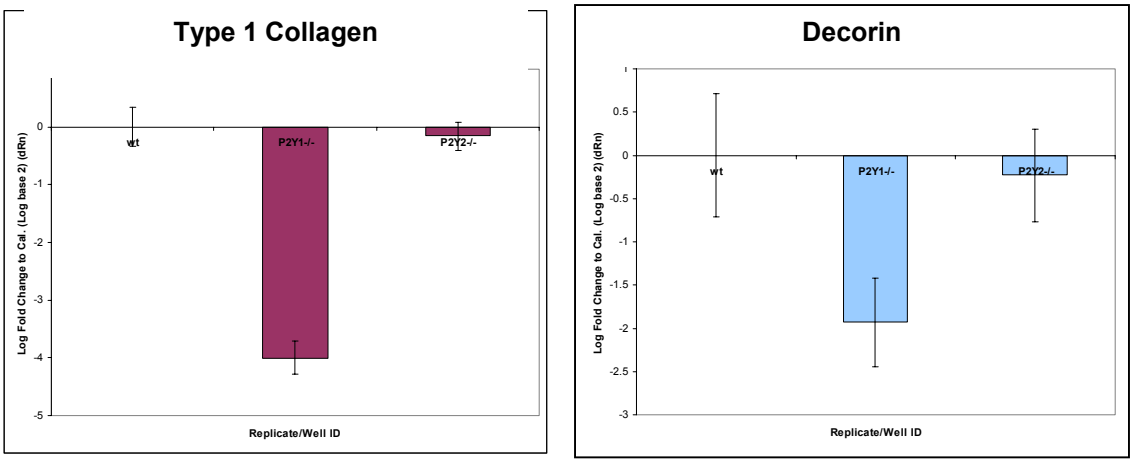

Figure 5.16 Evaluation of genes present in *wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) tendon revealed a decreased expression in $P2Y_1$(-/-) tendons in both type 1 collagen and decorin. This genetic decomposition provides insight as to why the mechanical inferiority was seen in $P2Y_1$(-/-) tendons.

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6 The Role of Purinoreceptors in Tendon Response to Stress

6.1 Abstract

In tendon cells, calcium waves are transmitted between cells via both a gap junction-mediated pathway and an extracellular, P2-receptor mediated pathway. Use of transgenic mice with $P2Y_1$ and $P2Y_2$ purinoceptor deficits provided a controlled phenotype to study the role of these receptors in whole tendon tissue and tenocyte responses to mechanical load. In addition, a continuous cell line was infected to overexpress the $P2Y_1$ and $P2Y_2$ receptors and evaluated for their ability to communicate a response to a mechanostimulus. Fura-2AM was used to quantitate $[Ca^{2+}]_{ic}$ values in cells responding to the increased Ca^{2+} of a single stimulated cell. Results indicate that ATP-responsive $P2Y_2$ (-/-) cells (with ADP-responsive receptors intact) have a dampened response to deformation whereas ADP-responsive $P2Y_1$ (-/-) cells (with ATP-responsive receptors intact) recruited more cells in the response to stimulation. Therefore, we hypothesized that activation of the $P2Y_2$ pathway may be involved in mechanical deformation signal transduction and amplification, whereas the $P2Y_1$ pathway may regulate signal dampening.

6.2 Introduction

Cells in mechanically active tissues detect, process, and relay load signals to surrounding cells in a feedback loop designed to provide tissue maintenance functions (Banes et al., 1995, 2001). Almost every cell type examined to date, including tenocytes, responds to mechanical loading with the release of ATP (Harden et al., 1997; Lazarowski et al., 1997; Cotrina et al., 1998; Watt et al., 1998; Weibe et al., 1999; Dixon et al., 2003; Bowler et al., 2001; Tsuzaki et al., 2005) ATP release occurs by transport through the ATP binding cassette proteins, one of which is the cystic fibrosis transmembrane regulator (CFTR), $P2X_7$ receptors, connexin hemichannels, or pannexin channels (Bao et al., 2004; Cotrina et al., 1998; Beigi et al., 1999; Schwiebert et al., 2000; Abraham et al., 1993). Extracellular ATP is a ubiquitous signaling molecule involved in such diverse cellular events as muscle contraction, vasodilation, neurotransmission, platelet aggregation, ion transport regulation, cell growth, cytokinesis, and inflammatory pathways (Abbracchio et al., 2006; Weber 1965; Moir, et al., 1973). Moreover, ATP is released by tenocytes and may play an essential role in tenocyte response to loads (Tsuzaki et al. 2005, 2003). Stresses induce ATP release in tendons and stimulate expression of IL-1β, COX 2, and MMP-3 (Tsuzaki et al. 2003). Hence, nucleotide release may serve as a negative feedback mechanism to prevent matrix remodeling, or more destructive pathways activated by these factors.

Purinoreceptors were first recognized by Burnstock in 1978, when he proposed two classes termed P1-purinoceptors, for which adenosine is the principal ligand, and P2-purinoceptors, recognizing both ATP and ADP (Burnstock, 1978). Furthermore, since their cloning in 1993, purinoceptors are classified into two

groups: a.) metabotropic (or G protein coupled) characterized by the P2Y class; or b.) ionotropic, with ligand-gated ion channels typical of P2X receptors (Communi et al., 2001; Abbracchio & Burnstock 1998; Burnstock & Kennedy, 1985). Pharmacological profiles, based on agonist potency order, transduction mechanisms, and molecular structure, have been established for eight distinct subtypes thus far: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Burnstock, 2007, Volonte et al., 2006; Ralevic & Burnstock, 1998).

The release of extracellular nucleotides in response to mechanical stimulation, and their involvement in calcium signaling via P2 receptors is well-known (You et al., 2002; Sauer et al., 2000; Homolya et al., 2000; Burnstock, 1997; Dubyak & El-Moatassim, 1993). Calcium is known to regulate tendon biology, and is one of the most rapid cellular responses to a mechanical stimulus (Bootman et al., 2001; Banes et al., 1995; Berridge et al., 1993). Cells from many tissues, including tendon, respond to mechanical stimuli by increasing intracellular calcium concentration ($[Ca^{2+}]_{ic}$) and releasing ATP to activate signaling pathways (Jones et al., 2005; Wall & Banes, 2005).

The increase in $[Ca^{2+}]_{ic}$ in stimulated cells is caused by a bi-phasic response involving an IP_3-dependent release of Ca^{2+} from internal stores, as well as an IP_3-dependent Ca^{2+} influx across the plasma membrane (Bertrand et al., 1990; Putney et al., 2007). Opening of the Ca^{2+} channels in the plasma membrane refills depleted Ca^{2+} stores in the endoplasmic reticulum and prolongs the Ca^{2+}-dependent signaling; (Putney et al., 2007). Communication to adjacent cells is then caused by IP_3 diffusing through gap junctions, resulting in a release of those internal calcium

stores in the neighboring cells (Evans & Boitano, 2001). As long as diffusion of IP_3 or Ca^{2+} ions continues, nearby cells will be recruited in the response, resulting in intercellular calcium waves.

Tenocytes respond to ATP via the metabotropic purinoceptors, particularly $P2Y_1$ and $P2Y_2$ types (Homolya, 2000; Yang, 2000; Francke, 1998; Gordjani, 1997). Furthermore, tenocytes express ecto-NTPases and actively degrade ATP into its hydrolyzed forms: ADP, AMP, and adenosine (Tsuzaki et al., 2005; Zimmerman et al., 2000). High concentrations of ATP and ADP likely occur in tendon tissue during trauma since lysed cells and platelets release millimolar quantities of ATP (Dubyak, 1991). Therefore, we suspect that ATP or one of its derivatives is an important load signal modulator and a candidate for involvement in tendinopathies.

Knockout mice provided a controlled phenotype to study the effects of the loss of $P2Y_1$ and $P2Y_2$ receptors in tendon. The association between ATP and $[Ca^{2+}]_{ic}$ provides a useful experimental design for evaluating the effects of nucleotides on cells. In the present study, we have evaluated this response by imaging a fluorophore that binds to calcium ions within cells, enabling real time quantitative data of the calcium wave propagation in response to mechanostimulatory or pharmacological events. Virally-infected MG63 osteoblast-derived cells overexpressing $P2Y_1$ or $P2Y_2$ receptors were examined to provide an additional perspective in understanding the roles of each receptor in the mechanical load response. Each cell type was examined to observe the effects of over-expressing these receptors on the calcium signaling response to mechanical indentation. We hypothesized that the loss of ATP-sensitive $P2Y_2$ nucleotide

receptors would result in a dose-dependent, threshold, or complete loss of a load response in tendon cells, gauged by altering mechanically-induced intercellular calcium signaling ($[Ca^{2+}]_{ic}$). Results indicate that ATP-responsive $P2Y_2$ (-/-) cells (with ADP-responsive receptors intact) demonstrated a dampened response to deformation whereas ADP-responsive $P2Y_1$(-/-) cells (with ATP-responsive receptors intact) recruited more cells in the response to stimulation. Therefore, we further hypothesized that activation of the $P2Y_2$ pathway may be involved in mechanical deformation signal transduction and amplification, whereas the $P2Y_1$ pathway may regulate signal dampening.

6.3 Materials and methods

6.3.1 Animals and experimental protocol

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. $P2Y_1$(-/-) and $P2Y_2$(-/-) mice initially in the B6D2 background (Homolya et al., 1999), were outbred in the 129SV background (Matos et al., 2005) with gene knockout confirmed by PCR. Age and sex-matched 129SV were used as wild type; three mice per group were used to achieve sufficient statistically significant data. Mice were sacrificed by CO_2 asphyxiation according to IACUC protocol.

6.3.2 Tendon harvest, cell isolation, and cell culture

Cells were harvested from the Achilles tendons of right and left legs of male, wild type (*wt*), $P2Y_1$ (-/-), and $P2Y_2$ (-/-) mice using sterile techniques (Figure 6.1).

At the time of sacrifice, male mice were between 8-10 weeks old. Tendon internal fibroblasts were isolated from Achilles tendons from *wt* and purinoceptor knockout mice ($P2Y_1$ (-/-), $P2Y_2$ (-/-)) by a modification of the trypsin/collagenase selective plating technique of Banes (Banes et al., 1988, 1995). Explant specimens were placed in a TissueTrap™ (Flexcell International Inc., Hillsborough, NC) outgrowth 6-well plate, which consists of a silastic hemicircle placed over half of each well in order to keep the explant in contact with the bottom of the well. The harvested cells were grown in Dulbecco's Modified Essential Media with high glucose (DMEM-H) (Gibco), 20% fetal bovine serum (FBS)(Equitech Bio, Kerrville, TX), and 1% penicillin/streptomycin/fungizone (PSF) until confluent in a 37° C, 5% CO_2, humidified environment.

Confluent cells of low passage from each isolation were released from culture plates by enzymatic treatment with 0.25% trypsin and seeded as micromass cultures on glass coverslips at a density of 4,000 cells/10µl for intercellular calcium imaging experiments. Cells in the spot cultures were allowed to attach to the substrate for one hour before the well was flooded with complete, serum-containing medium. Cultures were brought to quiescence by reducing the serum content from 20% to 2.5% over six days. For the ATP release experiment, 100,000 cells were plated in each well of a 6-well culture plate, for each isolation, and supplemented with complete media. Media were replaced on day 3.

6.3.3 pLXSN-HA-$P2Y_1$ and pLXSN-HA-$P2Y_2$ infection of MG63 cells

The immortalized MG63 human osteoblastic cell line was purchased from American Type Culture Collection (Manassas, VA). The pLXSN-HA-$P2Y_1$ and pLXSN-HA-$P2Y_2$ constructs were transfected into PA317 packaging cells. The virus was collected from the media of these cells and MG63 cells infected with the virus in the presence of 4μg/mL polybrene for 48 hours. Cells stably expressing $P2Y_1$ or $P2Y_2$ were selected by growth in 500μg/mL G418 for 3 weeks. MG63 infected with an empty vector were used as controls. Cells were cultured in MEM supplemented with 10% FBS (Sigma) and 1% PSF. Micromass cultures of 4,000cells/10μl were prepared for 1.) MG63 with an empty vector, 2.) MG63 overexpressing $P2Y_1$, and 3.) MG63 overexpressing $P2Y_2$, to be used in intercellular calcium signaling experiments. For the ATP release experiments, 100,000 cells were plated in each well of a 6-well culture plate, for each cell type, with MEM supplemented with 10% FBS and 1% PSF. Media were replaced on day 3.

To study the effects of the vectors on proliferation behaviors of these cells, a growth rate comparison was performed. Cells were plated at a density of 15,000 cells/well of a 24-well plate. Counts were performed each day at the same time for 7 days using a particle counter (Coulter). After enzymatic release with 0.25% trypsin, the cells from each well were counted in triplicate and averaged to obtain the mean number of cells/ml. The latter value was multiplied by the volume of cell suspension from each well to arrive at the number of cells present at each timepoint.

6.3.4 ATP release experiments

On day six, media were replaced with 3mL/well of serum-free, ATP-free, phenol red-free (phenol red acts as a confounding ATP mimic) DMEM-H (Gibco). Plates were returned to the incubator to sit undisturbed for at least 3 hours to allow ATP secretion to subside (Tsuzaki et al., 2003). Aliquots of 100µl were taken from each well prior to stimulation for control measurements. Each plate was rocked controllably six times to stimulate ATP secretion, and 100µl samples taken from the medium at 30 sec, 1, 2, 3, 4, 5, 10, 15 and 30 min after stimulation, and immediately placed on ice. After all samples had been collected, specimen tubes were placed in a 70°C heater block for five minutes to halt enzyme activity. Samples were stored at -20°C until luciferin/luciferase bioluminescence assay was performed.

6.3.5 Luciferin-luciferase bioluminescence assay

The ATP concentration was determined using the ATP Determination Kit according to the manufacturer's protocol (Invitrogen, USA). Media samples were prepared in a 96well microtiter plate. ATP standards of 0, 0.1nM, 1nM, 10nM, 100nM, and 1µm were prepared for each plate of samples. 100µl of a standard reaction solution containing recombinant firefly luciferase and its substrate, luciferin, were injected into each well within a microplate chamber of a Fluoroskan Ascent FL luminometer (Thermo Scientific, Waltham, MA); light emission (560nM at pH 7.8) was instantaneously measured 2 seconds after the reagent was injected into each well.

6.3.6 Pharmacology experiments

Adenosine trisphosphate (ATP) and adenosine diphosphate (ADP) (Sigma-Aldrich, USA) were reconstituted from powder as a 10mM stock solution stored at -20°C and dispensed in aliquots of 1mM working solutions. To confirm the activity of ATP, an exogenous bolus of the nucleotide was added to achieve final concentrations of 1 μM and 10 μM when added to the cells. To confirm that ATP was the modulator of the Ca^{2+} wave, non-hydrolysable analogs of the nucleotides, adenosine 5'-(3-thiotriphosphate) tetralithium salt (ATP-γS) and adenosine 5′-[β-thio]diphosphate trilithium salt (ADP-βS) (Sigma-Aldrich, USA), were reconstituted from powder and prepared as 1mM working solutions in EBSS with 20mM HEPES buffer (pH 7.2). From this working solution, 1μM, and 10 μM solutions were prepared for exogenous additions to cells following the same procedure for the standard nucleotides.

6.3.7 Intercellular calcium imaging and mechanical indentation

On day six, the cells in spot cultures were rinsed 2X with EBSS containing 20 mM HEPES, pH 7.2, 1.80 mM calcium chloride, and 0.8 mM magnesium sulfate (Tissue Culture Facility, Chapel Hill, NC). The cells were then loaded with a calcium fluorophore solution of 5μM Fura 2- AM with 0.10% pluronic (Molecular Probes, Junction City, Oregon) and incubated in the dark for 45-60 minutes at RT. The cells were then washed twice with calcium-containing EBSS to remove any unbound dye. (Boitano et al., 1995 ; Elfervig et al., 2001; Wall et al., 2004; Jones, et al., 2005)

The spot-culture glass cover slips were positioned on the stage of an Olympus BX-51 upright, fluorescence microscope (Opelco, Sterling, VA). A long

working distance, water immersion, 40X lens, CoolSnap® CCD camera (Roper Scientific, Trenton, NJ), and Sutter DG-4 wavelength switcher (SDR Clinical Technology, Sydney, Australia) with a 300W xenon light source were used to image Fura-2AM-loaded cells. RatioTool, an imaging software by Inovision (Research Triangle Park, NC) was used to monitor and calculate the $[Ca^{2+}]_{ic}$ in each cell. Fura-2AM fluorescence was collected for 60 seconds to establish a baseline measurement, followed by the controlled lowering of a 1μm-wide pipet tip onto the cell surface (Figure 6.2). Membrane indentation resulted in an intercellular calcium response that propagated to neighboring cells, creating an intercellular calcium ($[Ca^{2+}]_{ic}$) wave (Figure 6.3). $[Ca^{2+}]_{ic}$ values after the mechanical stimulus were calculated in order to establish the excitation of the poked cell as well as the degree of propagation of the Ca^{2+} transients to neighboring cells. Data were collected for at least 3 minutes post-stimulus. Calculations were made after comparison to a standardized Ca^{2+} calibration curve using the fluorometric ratio imaging technique (Grynkiewicz et al., 1985; Takahashi et al., 1999; Elfervig et al., 2001). Two trials per spot were performed on three spots per membrane from three independent cell isolations so that responses from 100-200 cells overall were analyzed statistically. A response was deemed significant if the increase in $[Ca^{2+}]_{ic}$ concentration was more than three standard deviations from the average of the 60-second baseline measurements (Elfervig et al., 2001). The value for the target cell was not included measurements nor in the number of responding cells.

6.3.8 Statistical analysis

Data were analyzed using SigmaStat (SPSS, Chicago, IL) and subjected to a Student's *t*-test, nonparametric *t*-test, or one-way ANOVA with a Dunnett's test, where appropriate, to determine significance between and among groups. The average number of cells that responded to mechanical stimulation was determined by dividing the total number of cells that responded by the number of mechanical stimulation events per field of view. The mean peak Ca^{2+} value was calculated by averaging the Ca^{2+} increase for only the responding cells. (Elfervig et al., 2001, Jones et al., 2005) All results were expressed as the mean ± standard error, unless otherwise indicated.

6.4 Results

6.4.1 Virally infected cells growth study

A cell growth study quantified the lag, log, and stationary phases of virally infected cells, and did not illuminate any disparities between MG63 control cells and those infected with empty vectors, $P2Y_1$ constructs, or $P2Y_2$ constructs (Figure 6.4).

6.4.2 ATP release experiments

Wt and $P2Y_1$(-/-) tenocytes responded to fluid shear stress by releasing ATP (Figure 6.5). ATP levels released into the media reached their maximum values by 1 minute (wt = 22.41+/-5.06 SE; $P2Y_1$(-/-)=25.71+/- 5.19 SE), and the effects of ecto-NTPases were evident as the ATP concentration decreased over 30 minutes. In contrast, Figure 6.5 shows the $P2Y_2$(-/-) cells did not show a significant release of ATP in response to the stimulus (max=5.06+/-0.63 SE). The differences between

ATP concentrations for media collected from $P2Y_2$ (-/-) cells were significantly different from values from *wt* and $P2Y_1$(-/-) ($p<0.001$).

ATP release data did not show any significant differences between groups of virally infected cells that over-expressed either receptor (Figure 6.6). Generally, ATP levels rose in response to fluid shear stimulation, and began to decrease after just one minute post-stimulation. This indicates that ecto-NTPases are present and functioning.

6.4.3 Pharmacology experiments

As a positive control, tendon cells were exposed to a bolus of ATP, ADP, ATP-γS, and ADP-βS added to the saline solution, to achieve final concentrations of 1 or 10μM. A robust response to 1μM ATP was evident in *wt* and $P2Y_1$(-/-) cells, while those without the ATP sensitive receptor ($P2Y_2$(-/-)) lacked a significant response (Figure 6.7). A significant response was not detected in any group when ADP was added.

In order to discount the effects of metabolites (ADP→ADP + 1P→AMP +2P→Adenosine +3P), non-metabolyzable analogs of the nucleotides were used. ATP-γS and ADP-βS were added to cells to confirm the effects noted for the above exogenous additions (Figure 6.8). These results confirmed that the principal activity of ATP acting on$P2Y_2$ receptors was the primary stimulus in the calcium response to exogenous nucleotides. ADP did not incite a signaling event.

6.4.4 Mechanical indentation of tenocytes

A target cell was deformed by pipette indentation of the plasma membrane. There was an immediate rise in the intracellular calcium concentration of the target cell, followed by a prompt recruitment of neighboring cells in a propagation of an intercellular calcium wave (Figure 6.9). The baseline calcium measurements were not significantly different between or among cell types ($p < 0.001$), but the response was significant in each of the cell types examined ($p < 0.001$) (Figure 6.10).

The ability of cells to communicate the stimulus event to neighboring cells is critical for normal cellular function. The percentage of cells responding to stimulation of the target cell was significantly different between groups ($p<0.001$), with $P2Y_1$(-/-) tenocytes indicating an increased intercellular communication and $P2Y_2$(-/-) showing a decreased ability to transmit the response to neighboring cells (Figure 6.11). In addition to degree of recruitment, it was also noted that the response of the neighboring cells lacking the ATP-sensitive $P2Y_2$ receptor led to higher intracellular calcium values as compared to $P2Y_1$(-/-) cells. The mean peak $[Ca^{2+}]_{ic}$ response was significantly greater than the baseline calcium value for each cell type ($p< 0.001$).

In addition, upregulation of $P2Y_1$ and $P2Y_2$ receptors had a significant impact on the behavior of cells in response to a stimulus. The mean peak values of these cells was shown in Figure 6.12; these values are statistically different ($p < 0.001$), as was the percent of cells responding to the mechanical indentation (Figure 6.13).

6.5 Discussion

These are the first results suggesting an interplay of ATP and ADP in regulating the degree of mechanosensitivity in connective tissue cells. A cell must be able to respond, then desensitize to a mechanical stimulus. However, the factors involved in this attenuation response are not currently well studied. In the present studies involving intracellular calcium signaling in *wt*, $P2Y_1$(-/-), and $P2Y_2$(-/-) tenocytes, ATP appears to be a stimulatory signal that increases signal transduction to load, while ADP acts as an antagonist that dampens the response (Figure 6.8). Study of these pathways may help unravel the complex and interdependent pathways that regulate sensitivity to mechanical load, and reveal how nucleotides modulate these responses. It is postulated that this nucleotide pair represents a feedback control system to modulate a load response that negatively controls the magnitude of mechanical signaling.

Tenocytes express purinoceptors and respond to ATP and other nucleotides and nucleosides (Francke et al., 1998; Banes et al., 2001; Jones et al., 2005). Moreover, mechanical load induces ATP secretion in tendon cells (Tsuzaki et al., 2003). Thus, ATP release is an important modulator of mechanical load responses in tendon cells and is a useful standard to observe. Results of the ATP secretion assay indicate mouse tenocytes lacking $P2Y_2$ receptors fail to secrete significant levels of ATP in response to shear stress. This indicates a possible developmental connection between the conduits for ATP release and the receptors activated by the molecule. Another consideration not investigated in this experiment is the native levels of ecto-NTPases and activities. It is possible that high levels of these

nucleotide-degrading enzyme may result in patterns similar to those in our $P2Y_2$(-/-) data.

Since tissues often express multiple P2 receptor subtypes, it can be difficult to assign a receptor to a specific function (Boarder, 1998; Homolya, 2000). In the present studies, a P2Y-knockout mouse model was used, in which the gene for the $P2Y_1$, or $P2Y_2$ receptor was mutated and rendered nonfunctional. These two receptor subtypes have often been implicated in nucleotide-mediated calcium signaling. The pharmacological profiles of these receptors are well known (Burrell, 2003; Wiebe, 1999; Homolya, 1999). Nucleotides and their non-metabolyzable analogs were used to identify the receptors responsible for the transmission of the calcium wave in response to mechanical stimulation. As expected, the $P2Y_1$(-/-) cells demonstrated a hypersensitivity to exogenous addition of ATP and ATP-γS, while lacking a response to ADP and ADP-βS as well. This interaction further supports the hypothesis that ATP is a stimulatory signal tempered by its metabolite ADP. Likewise, the null response of $P2Y_2$ (-/-) cells to ATP, and ATP-γS indicated that these receptors are principle sensory components at play in this event.

Graphs of *wt*, $P2Y_1$(-/-) and $P2Y_2$(-/-) cells showed the poked cell responded to the micro-indentation by increasing intracellular calcium, indicating that voltage-regulated calcium channels were present and opened (Figure 6.9). However, in the $P2Y_2$ (-/-) cells, only 1-2 neighboring cells responded, indicating a decreased sensitivity or lack of intercellular communication. A contrasting response was evident with the $P2Y_1$ (-/-) cells, as these were hyper-reactive in recruiting more cells to participate in the response. These data indicate that the $P2Y_2$ receptor may play

a positive role and the $P2Y_1$ receptor may modulate cell recruitment in the calcium response to applied strain. Even in $P2Y_2$(-/-) tenocytes, the target cells always responded; however, neighboring cells did not always participate in the response. Therefore, purinoreceptor activation is associated with cell recruitment and cell-cell communication. The details of this mechanism, however, have not been elucidated.

Interestingly however, the Ca^{2+} signaling response of $P2Y_1$(-/-) cells lasted longer than in other groups (Figure 6.9). This underscores the importance of ADP activation of $P2Y_1$ receptors to blunt the $[Ca^{2+}]_{ic}$ response. Likewise, if ATP is secreted, other cells in the vicinity of the target cell may also signal to the stimulus in a paracrine manner. Although the molecular mechanism is not well defined, purinoreceptor activation and Ca^{2+} wave propagation may by mediated by ATP release from mechanically stimulated cells (Sauer et al., 2000). Thus, scarcity of ATP secretion, evident in $P2Y_2$(-/-) tenocytes, may be associated with an inability to communicate the signal to neighboring cells. However, it should be noted that this is only one method of intercellular calcium wave propagation. Another, the diffusion of IP_3 through gap junctions, is also responsible for communication between cells (Berridge, 2000). It is likely that connected *wt*, and $P2Y_1$(-/-) cells received IP_3 via gap junctions as the biphasic increase in $[Ca^{2+}]_{ic}$ was induced. However, this transfer may not have occurred between $P2Y_2$(-/-) tenocytes.

The responses of virally infected osteoblast-like cells were unexpected. It was hypothesized that cells overexpressing $P2Y_1$, the proposed negative modulator, would either not respond or may have a less robust signal. On the other hand, a more robust response was expected in $P2Y_2$ overexpressing cells, as this was the

suggested stimulatory pathway. Experimental data indicate these hypotheses to be false. Be that as it may, the upregulation of specific genes has been shown to have unpredicted and sometimes unapparent consequences. On the other hand, it is possible that $P2Y_1$ and $P2Y_2$ receptors may play a role in development of mechanotransduction machinery, which in the uncharacterized knockout animals, are not present.

When two receptors are co-expressed in a given tissue, cross-talk often occurs (Czajkowski, 2002; Werry, 2003). Cross-talk can either enhance the Ca^{2+} signaling response in cells or cause desensitization of the receptors. The $P2Y_1$ receptor is more potently coupled to ADP, while the $P2Y_2$ receptor has a greater potency for ATP (Ralevic, 1998). When both nucleotides are released, they may compete at the same receptor, whereby one nucleotide may induce a release of Ca^{2+} from intracellular stores, while the second nucleotide inhibits this release (Sabala, 2001; Park, 1997). Furthermore, ATP has been shown in several cell types to have an antagonistic effect at the $P2Y_1$ receptor (Boyer, 1996; Ralevic, 1998). In the wild type cells, both the $P2Y_1$ and $P2Y_2$ receptors were present.

An important modulatory function of extracellular nucleotides has been demonstrated in tendon cells. Loss of P2 purinoreceptors negatively influences the spread of intercellular calcium transients, highlighting the fundamental importance of purines in tenocyte response to load. We conclude from the results of this study that the $P2Y_2$ receptor is a necessary component in the calcium signaling response of tenocytes mediated via the paracrine release of ATP. This class of receptors also has a primary role in the control of sensitivity to strain and mechanotransduction in

tendon cells. This study emphasizes a role for nucleotides in the ability to temper load response in tendons. Functional tissue engineering of connective tissues will rely on the ability to control factors that contribute to mechanical integrity, such as load response. Genetic alteration of purinoreceptor expression or the use of non-metabolyzable nucleotides to alter the sensitivity of tenocytes to load may confer the ability to control these parameters. Clinical significance follows with the potential to use of ATP, ADP, or non-metabolyzable analogs for tailored drug therapy for the treatment of damaged tendons.

6.6 Acknowledgements

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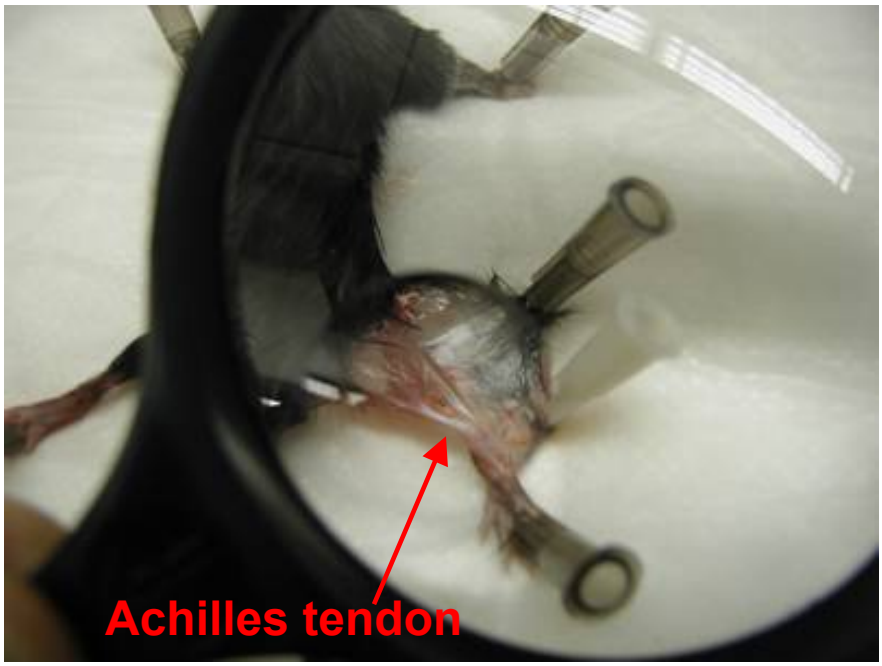


Figure 6.1 The Achilles tendons of *wt*, $P2Y_1(-/-)$ and $P2Y_2(-/-)$ mice were harvested as explant tissues. Cells migrated from the tissue onto culture dishes and were available to be used in a variety of experiments.

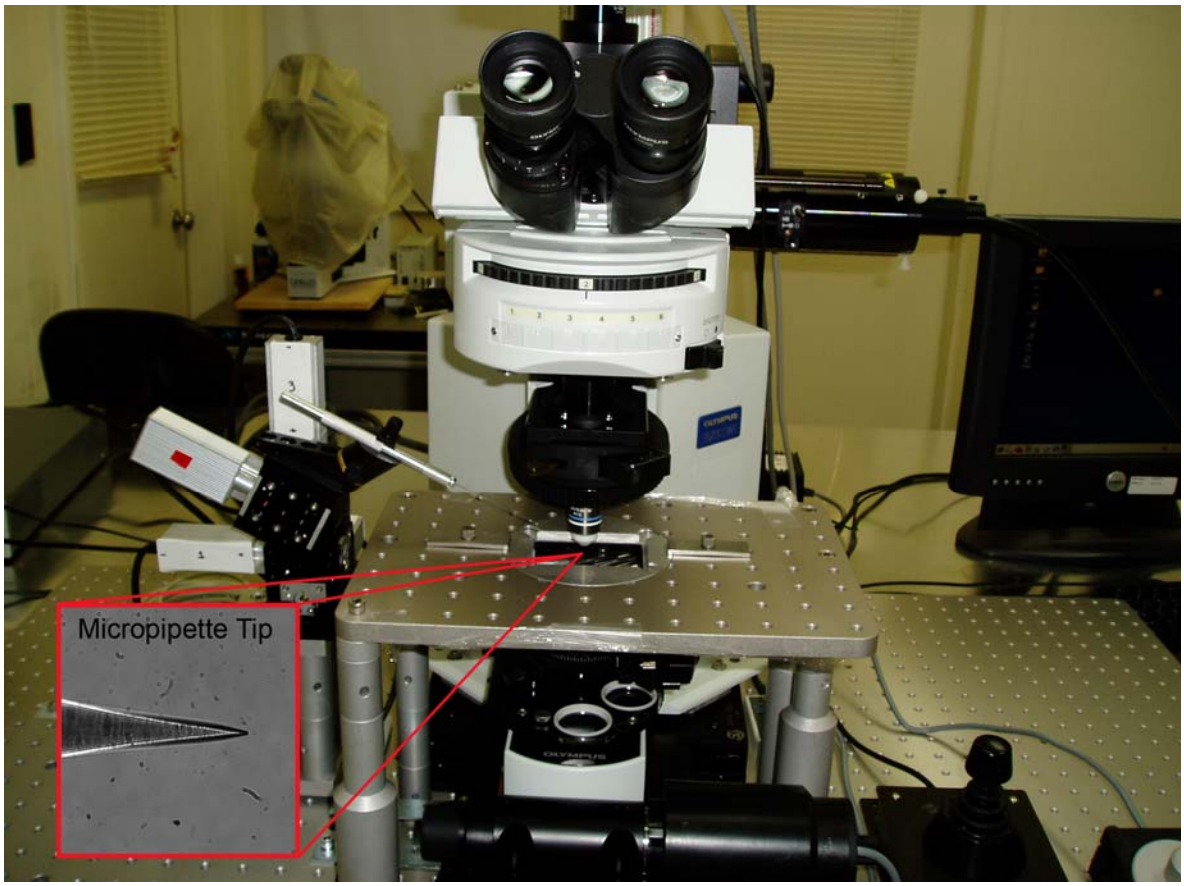


Figure 6.2 A micropipette was maneuvered by a computer controlled multi-axis stepper below an objective. A filter was in place to collect emissions from fura-2Am loaded cells so that real time, quantitative calcium concentrations can be monitored.

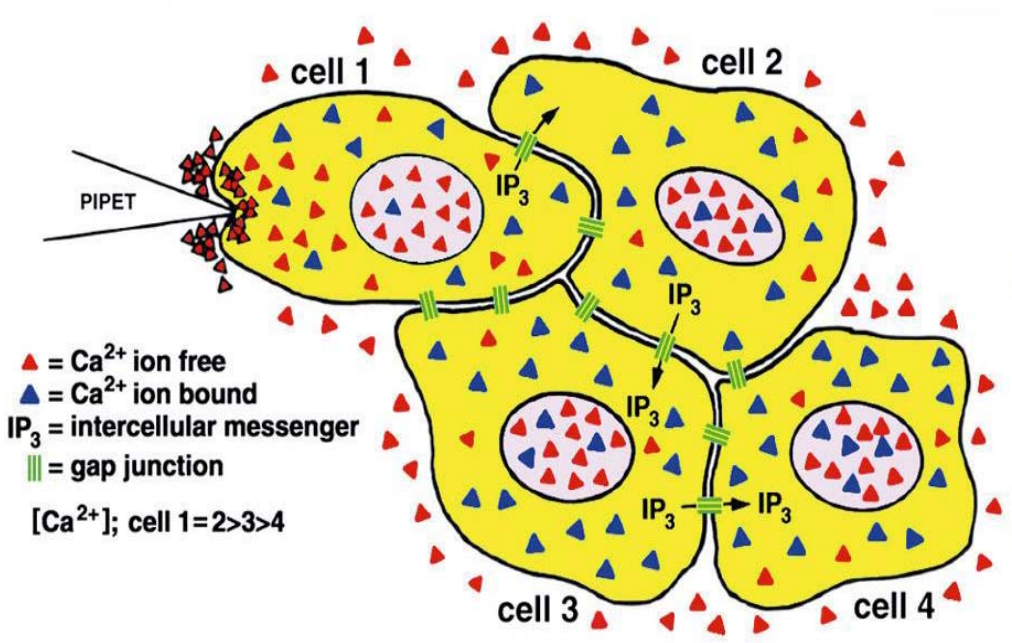


Figure 6.3 In the event of a mechanical 'poke', the target cell responds with a bi-phasic increase in calcium. This response is mediated by calcium channels on the cell membrane, internal calcium stores, and second messengers. When gap junctions are present, some of the second messengers, IP_3 in particular, can travel to adjoining cells and stimulate a subsequent calcium response. In this way, a stimulus effecting one cell can be communicated to other nearby cells.

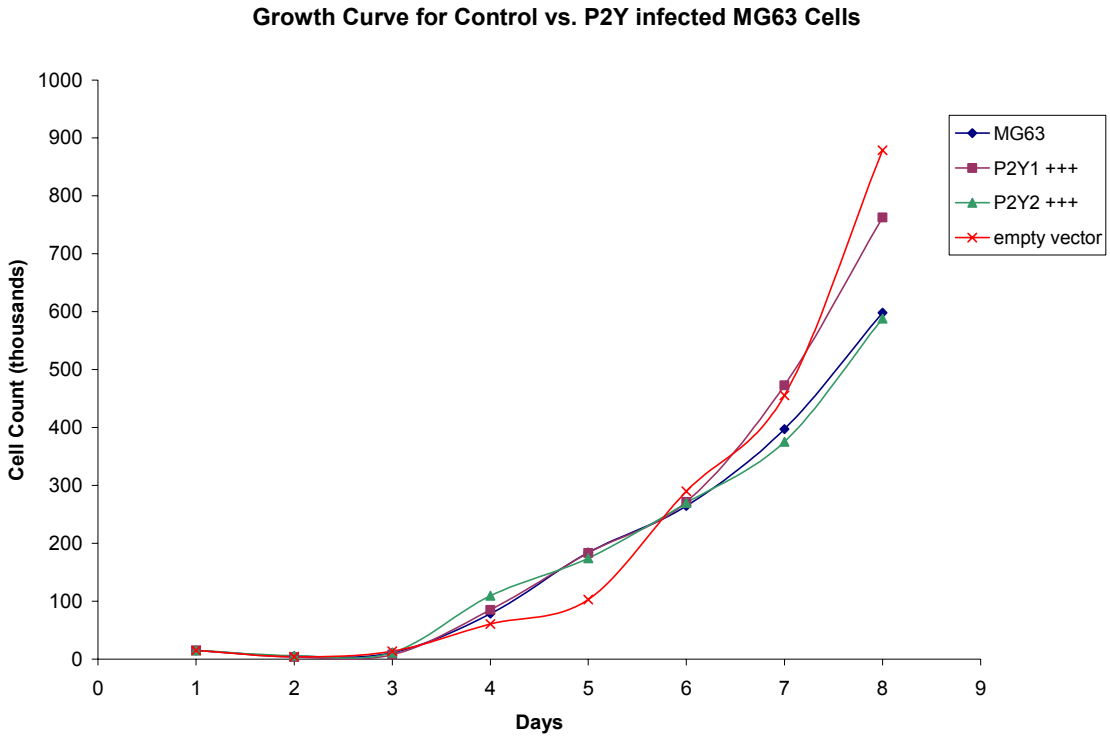


Figure 6.4 A growth control study was used to examine the growth patterns of infected vs. control cell types. No significant difference was observed. ($p<0.05$)

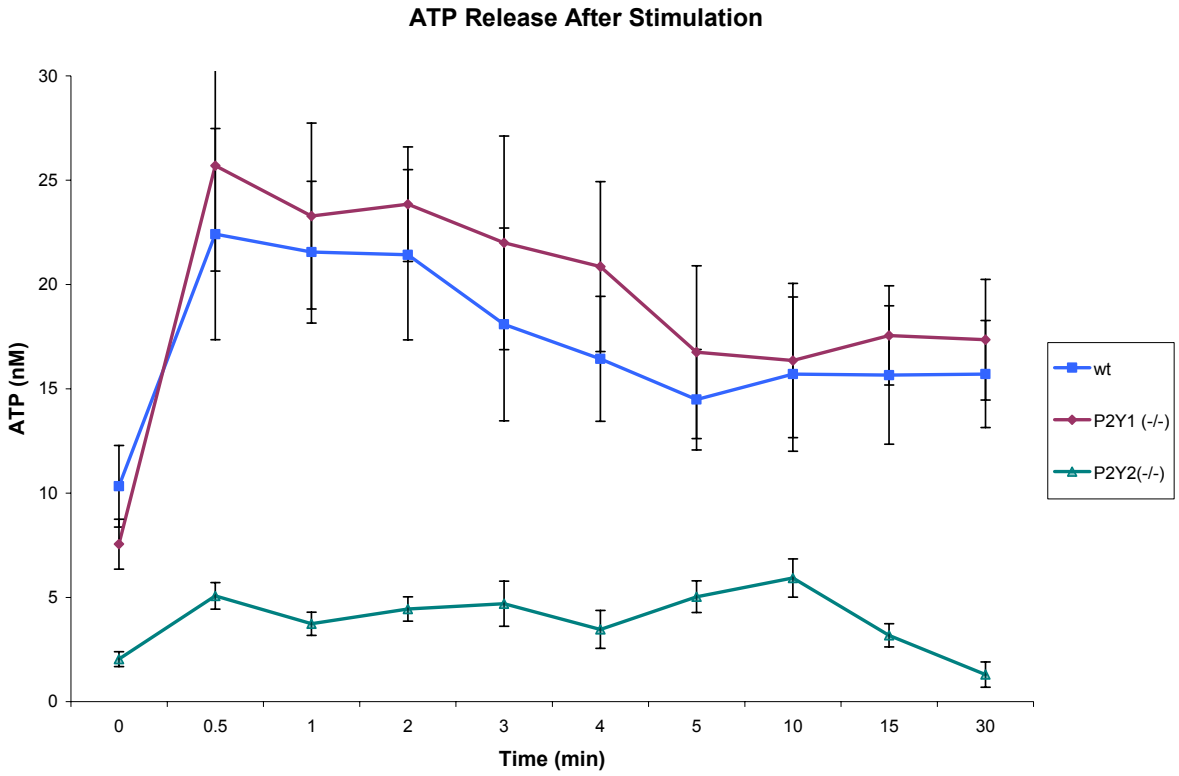


Figure 6.5 ATP secretion from wt and knockout cells was measured following a fluid shear stimulus. Data demonstrated a markedly lower concentration in the media of $P2Y_2(-/-)$ cells.

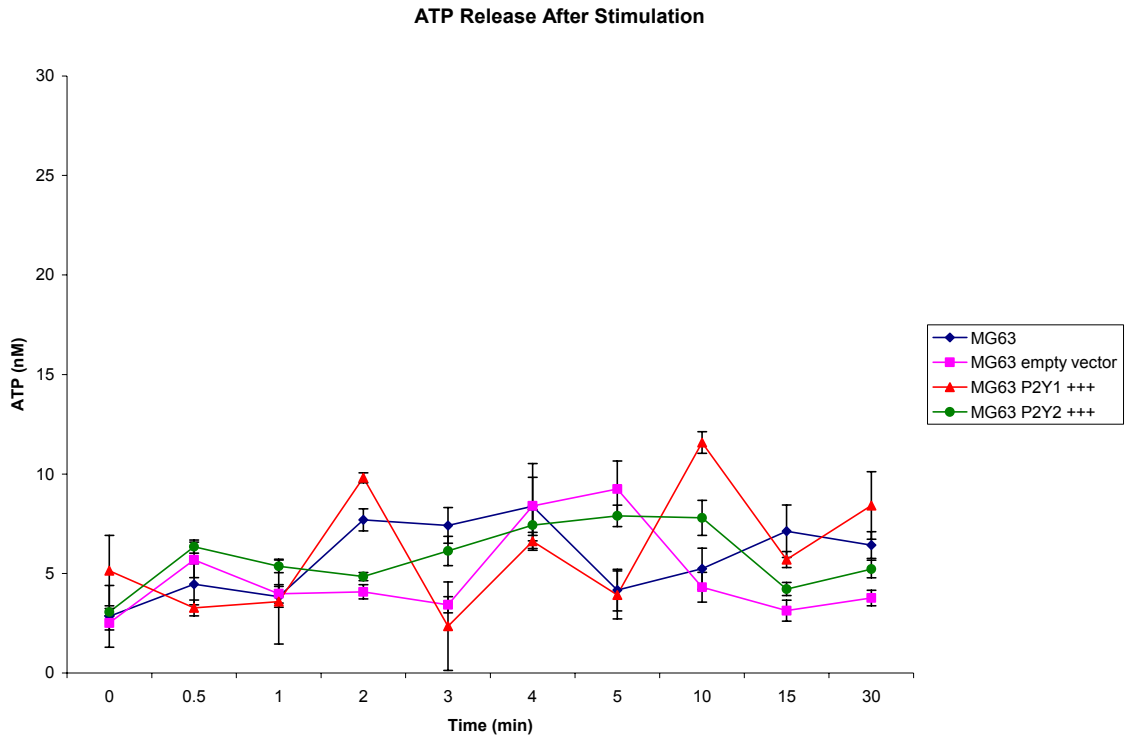
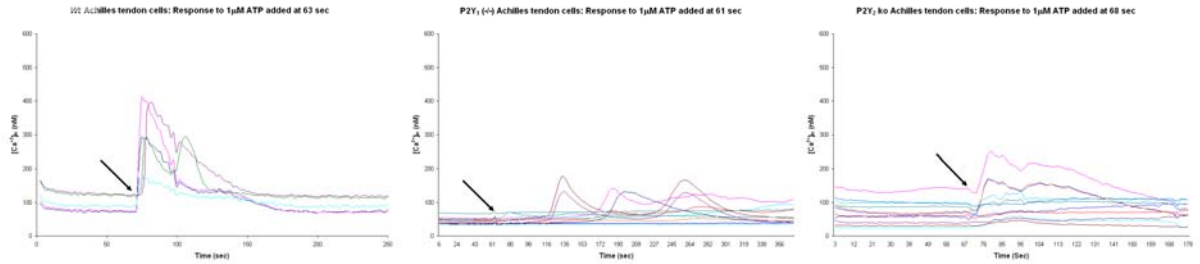


Figure 6.6 ATP release from control and infected cells did not show a significantly different pattern between cell types ($p<0.05$)).

a.)



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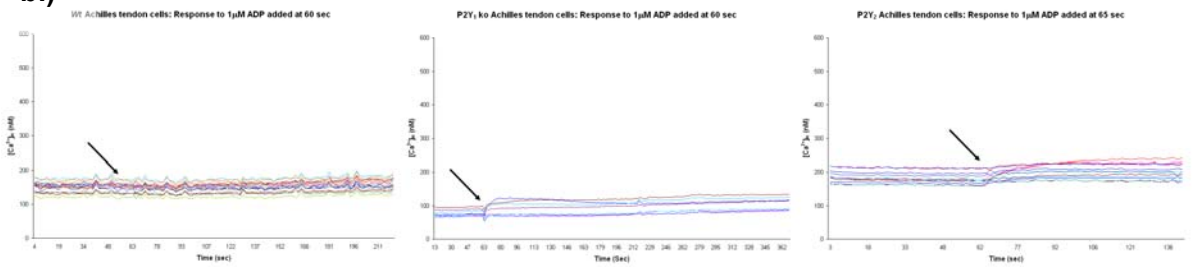

Figure 6.8 Graphs of intracellular calcium levels tracking murine *wt*, $P2Y_1$(-/-) and $P2Y_2$(-/-) tenocyte response to stimuli. Each line represents a single cell; the arrow depicts the time at which a.) an extracellular bolus of 1μM ATP and b.) 1μM ADP was added to cells. Each line depicts a single cell. A response was recognized in a neighboring cell if the intracellular calcium concentration elevated to more than three-times the standard deviation of the 60 sec baseline average.

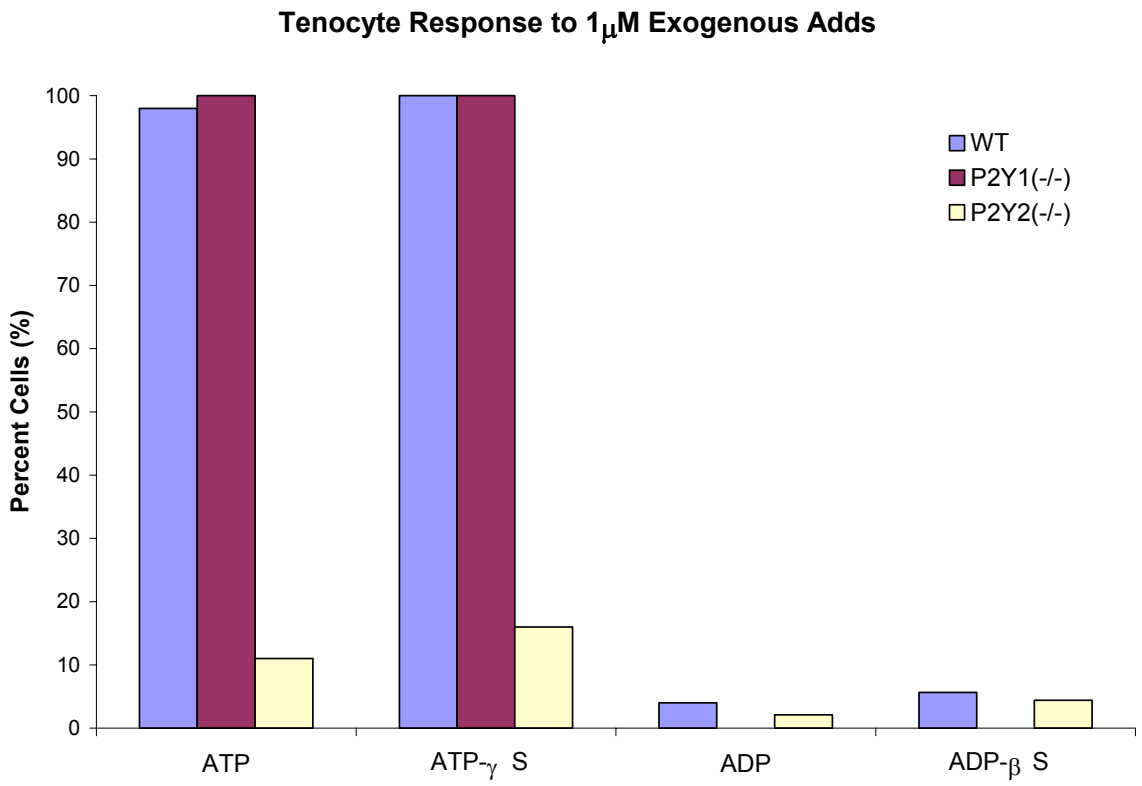

Figure 6.7 As a positive control, *wt* and knockout tenocytes were exposed to a bolus of ATP, ADP, ATP-γS, and ADP-βS. ATP and it's non-metabolyzable analog evoked a calcium response with just 1μM in *wt* and $P2Y_1$(-/-) (in which ATP receptors were present). ADP and ADP-βS did not invoke a significant response in any cell types.

Figure 6.9

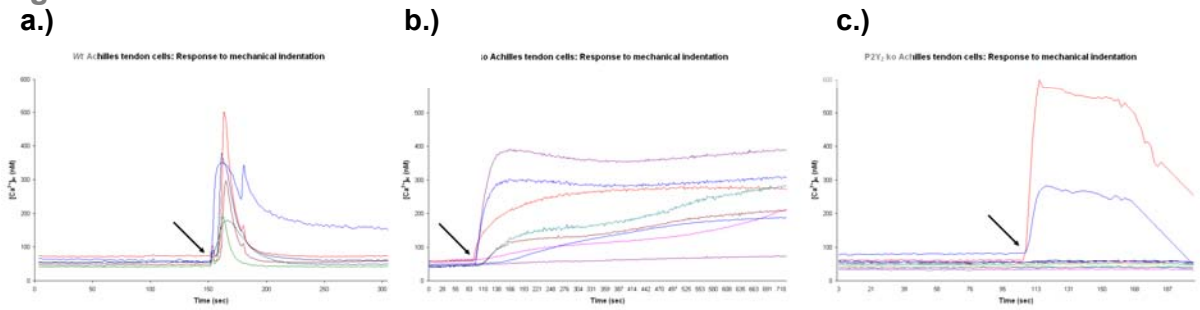

Figure 6.10 Graphical data of **a.)** *wt*, **b.)** $P2Y_1(-/-)$, and **c.)** $P2Y_2(-/-)$ responding to mechanical indentation. Each line represents the $[Ca^{2+}]_{ic}$ concentration of a single cell. A 60-second baseline measurement was taken before the pipet tip was lowered onto a single target cell, indicated by the arrow. The event was followed by the spread of elevated calcium levels within neighboring cells. $P2Y_1$ single knockout cells respond in a hypersensitive manner compared to *wt*, whereby calcium levels remain elevated for a longer period of time, and more cells are recruited in the response, than *wt*. $P2Y_2$ (-/-) cells show a response pattern indicating the signal is not effectively communicated to neighbors with a decreased cell number communicating the increase in Ca^{2+}.

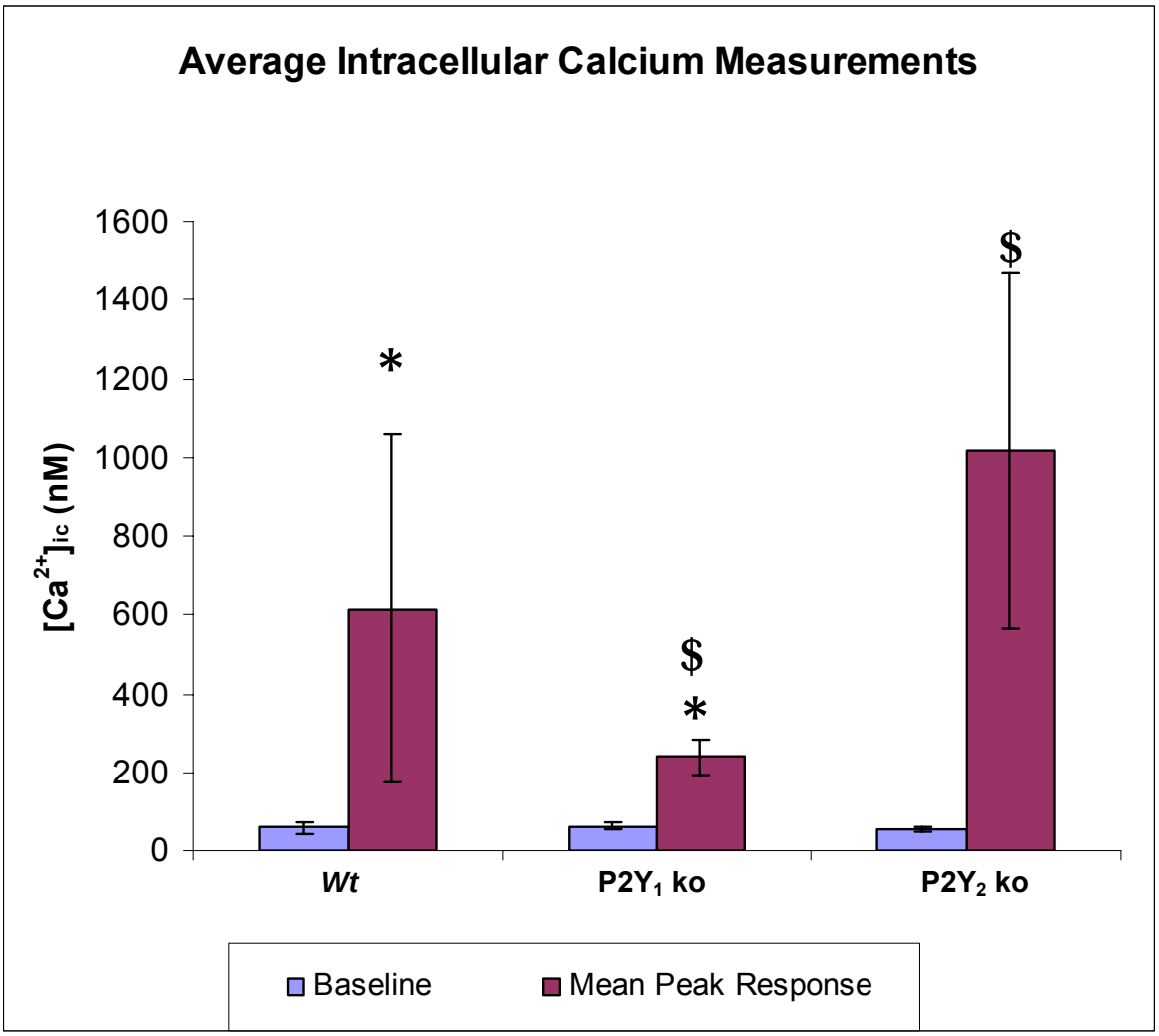

Figure 6.11 Intracellular calcium levels of *wt*, $P2Y_1$(-/-), $P2Y_2$(-/-) Achilles tendon cells before and after the pipet tip was lowered onto a single target cell. The mean peak response signifies the mean transmission of elevated intracellular calcium levels in the neighboring cells within the field of view following the stimulation event. ("*"=p<.001)

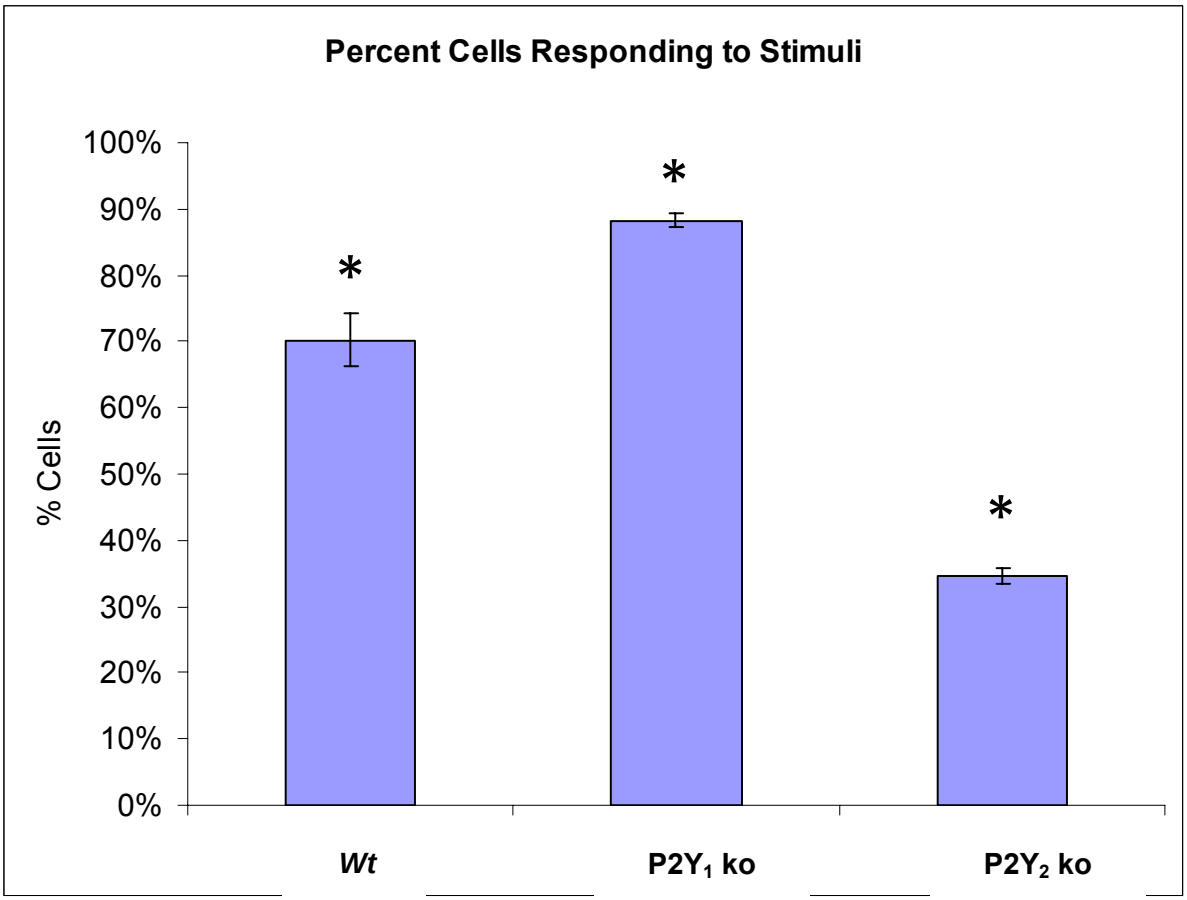

Figure 6.12 The average percent of cells, not including the poked cell, that responded to the stimuli showed a significant difference between *wt* and $P2Y_1$(-/-), *wt* and $P2Y_2$ (-/-), and $P2Y_1$(-/-) and $P2Y_2$(-/-) Achilles tenocytes. ("*"=p<.001)

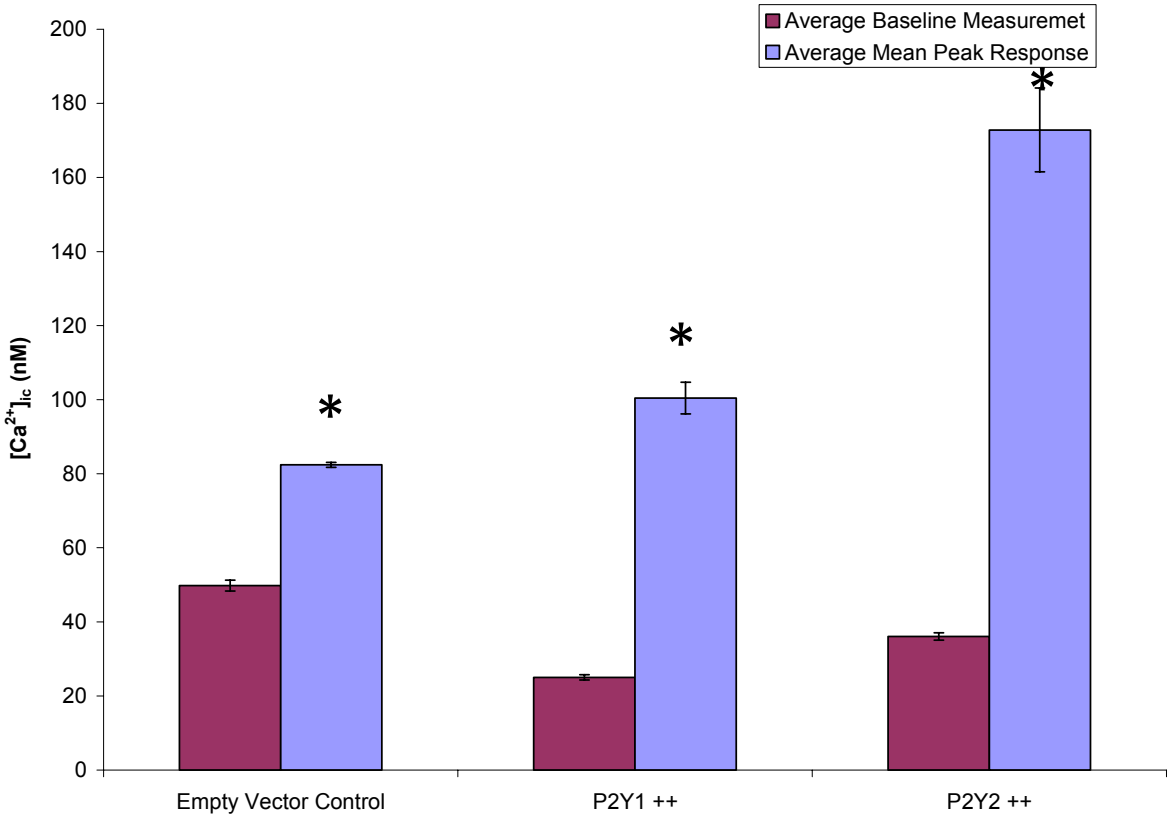

Figure 6.13 The mean peak response to mechanical indentation in $P2Y_1$ and $P2Y_2$ upregulated MG63 cells. A significant difference was demonstrated between the response in MG63 and empty vector controls and the cells with upregulated expression of the purinoreceptors. ("*"=$p<0.001$)

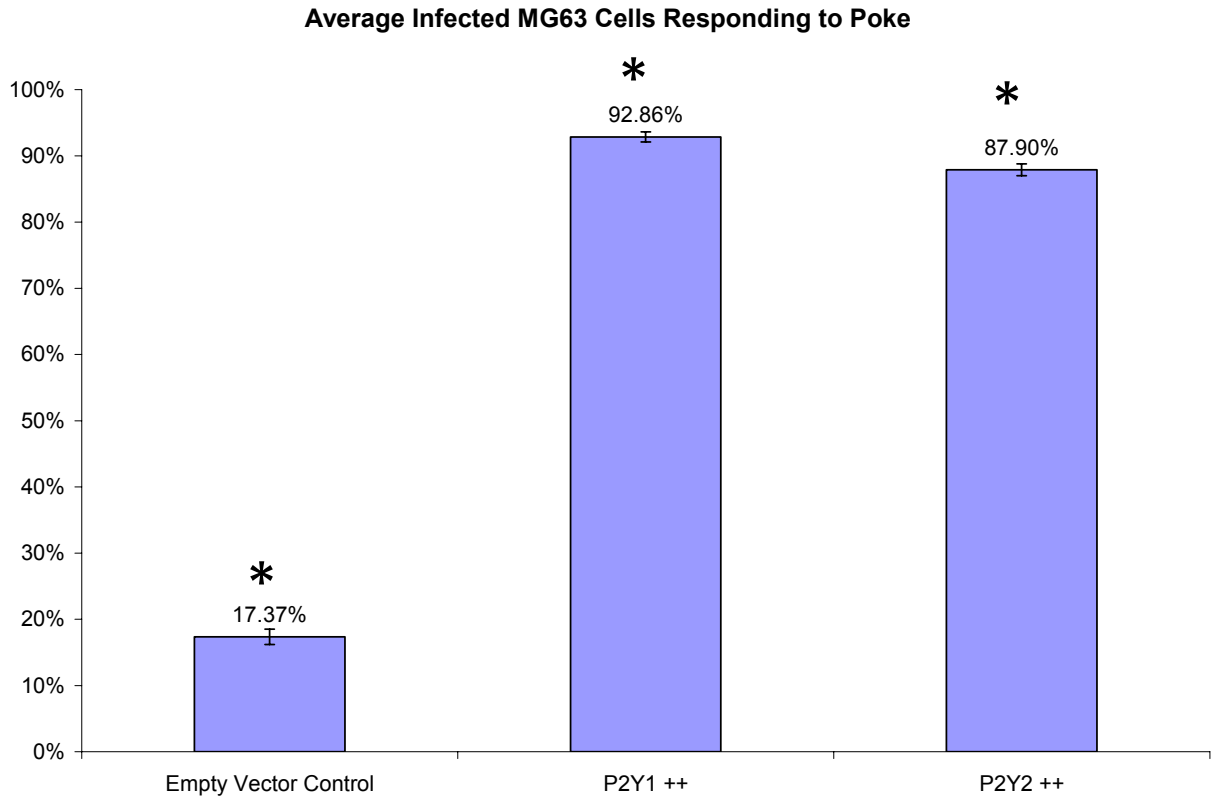

Figure 6.14 Average percent of $P2Y_1$ and $P2Y_2$ upregulated MG63 cells responding to mechanical indentation. The overexpression of both $P2Y_1$ and $P2Y_2$ had an effect on the degree of recruitment in the response. ("*"=$p<0.001$)

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7 The Effects of Purinoreceptor Deficits on Connexin 43 Localization

7.1 Abstract

Gap junctions are specialized intercellular junctions characterized by their ability to modulate various biological processes through mediating the passive movement of ions and second messengers. They are a critical part of intercellular communication, as are purinoreceptors. Immunostaining *wt* and purinoreceptor knockout tenocytes for Cx43, a key gap-junction forming protein, showed an altered location patterning within $P2Y_2$(-/-) tenocytes. This would indicate a possible mechanism for the decreased communication of a response when a target cell is stimulated.

7.2 Introduction

Tendon cells exist in a mechanically active environment. To respond to the daily loads imposed on tissue, tenocytes must coordinate an appropriate response to the mechanotransduction signaling events that result from loading. It has been shown that tendon cells can propagate a calcium wave in response to mechanical load stimuli via gap-junctional coupling (Banes et al., 1995; Wall et al., 2005; Wall & Banes 2005; Jones et al., 2005). The calcium signaling cascade involves diffusion of IP_3 through gap junctions, particularly connexin 43 (Cx43) in tenocytes, to initiate

a response in adjoining cells (McNeilly et al., 1996; Wall & Banes, 2005). Moreover, connexin hemichannels are mechanosensitive and permeant to ATP, although the mechanism is unknown (Cotrina et al., 1998; Bao et al., 2004).

Connexin monomers are transported to the plasma membrane, where they assemble as a hexamer with a central pore. Here, these proteins create a hemichannel that can associate with hemichannels on adjacent cells to form cell-cell channels. Clusters of these channels assemble to make gap junctions. Gap junction communication is important in development and regulation of cell growth and maintenance (Musil et al., 1990; McNeilly et al., 1996). Tenocytes *in vivo* and *in vitro* express Cx43, and respond to growth factors and mechanical stimuli change phosphorylation state and gene regulation, respectively (Banes et al., 1995, 1996; McNeilly et al., 1996).

Connexin proteins have been shown to interact with a diverse array of proteins, which are likely to regulate connexin assembly, trafficking, turnover, channel gating, and may modulate function in response to physiological stimulation and pathological conditions (Duffy et al., 2002; Thomas et al., 2002; Herve et al., 2004). Phosphorylation of Cx43 is an important modulatory event to control the assembly and function of gap junctions, whereby the channel transforms into a closed form thereby decreasing intercellular communication (Musil & Goodenough, 1991; Lampe et al., 2000; Giepmans et al., 2001; Lin et al., 2001). In cultured cells, the half-life of cells has been reported to be 1.5-4 hours (Musil et al., 2001). Two major pathways for the degradation of connexins have been described, through the lysosome and the proteasome. The lysosomal pathway degrades endocytosed

material including membrane proteins, and the proteasomal pathway is the route of degradation for cytosolic and nuclear proteins (Salameh et al., 2006). The short half-life determined for connexins has been used to argue that the degree of intercellular communication between cells may be regulated by changes in synthesis or degradation rates.

Suadicani et al., showed $P2Y_2$ receptor expression was altered in Cx43 null astrocytes (Suadicani et al., 2003). Moreover, a large number of genes associated with intercellular communication were up- or down-regulated in the knockout cells (Goldberg et al., 2000; Moorby & Patel, 2001; Liu et al., 2001). Interestingly, not all of the genes that changed in Cx43(-/-) astrocytes are required for the formation of functional gap junction channels (Iacobas et al., 2003). This indicates there may be a direct feedback involving Cx43 or its binding partners to activate other genes (Iacobas et al., 2003).

Tenocytes *in vivo* align in longitudinal rows separated by collagen fibrils, and form a multidimensional network of cell processes interweaving through the extracellular matrix (ECM). Cx43 has been immunohistochemically detected at the end of these processes, ultimately linking cells within the 3D matrix of a tissue. (McNeilly et al., 1996) Furthermore, Cx43 has been associated with actin localization within these processes, which may aid in trafficking and docking of these junctions at cellular peripheries extending toward neighboring cells. (Lauf et al., 2002; Wall et al., 2007)

Intercellular communication between cells can be achieved through a variety of mechanisms. One such way, used by almost every cell type in a wide variety of

cellular processes, is calcium signaling. Cellular activities involved with the initiation, maintenance, and cessation of life, are coordinated by the transmission of elevated concentrations of intracellular calcium, resulting in a wave propagation that spreads from cell to cell. This intercellular calcium wave can come about in two ways. First, gap junctions that physically, chemically, and electrically connect adjacent cells, allow the passage of IP_3 (Boitano et al 1992; Sneyd et al., 1995; Banes et al., 1995; McNeilly et al., 1996; Sanderson 1995; Bruzzone, 2001; Wall & Banes 2005). Secondly, activation of purinoreceptors via their agonistic nucleotides initiates an IP_3-dependent signaling in a paracrine manner.

Connexin expression is modulated by mechanical stimulation (Banes et al., 1999). Thus, there may be an association between mechanosensitivity and connexin activity. In previous studies, mouse tenocytes lacking the ATP-sensitive $P2Y_2$ receptors have shown a muted response to mechanical stimulation (See Chapter 6). It was hypothesized that $P2Y_2$(-/-) tenocytes failed to communicate a response to a membrane indentation to neighboring cells because of inadequate gap junction coupling. It is unknown if there exists an association between purinoreceptors and connexins; however, both mechanisms are key players in mechanotransduction events. Therefore, it is important to understand the interplay between nucleotide receptors and gap junctions.

7.3 Materials and methods

7.3.1 Animals and experimental protocol

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. $P2Y_1$(-/-) and $P2Y_2$(-/-) mice initially in the B6D2 background (Homolya et al., 1999), were outbred in the 129SV background (Matos et al., 2005) with gene knockout confirmed by PCR. Age and sex-matched 129SV were used as wild type; three mice per group were used to achieve sufficient statistically significant data. Mice were sacrificed by CO_2 asphyxiation according to IACUC protocol.

7.3.2 Cell isolation and culture

Cells were harvested from the Achilles tendons of right and left legs of male, wild type (*wt*), $P2Y_1$ (-/-), and $P2Y_2$ (-/-) mice using sterile techniques (n=3, each). At the time of sacrifice, male mice were between 8-10 weeks old. Tendon internal fibroblasts were isolated from Achilles tendons from *wt* and purinoceptor knockout mice ($P2Y_1$ (-/-), $P2Y_2$ (-/-)) by a modification of the trypsin/collagenase selective plating technique of Banes (Banes et al., 1988, 1995). Explant specimens were placed in a TissueTrap™ (Flexcell International Inc., Hillsborough, NC) outgrowth 6-well plate, which consists of a silastic hemicircle placed over half of each well in order to keep the explant in contact with the bottom of the well. The harvested cells were grown in Dulbecco's Modified Essential Media with high glucose (DMEM-H) (Gibco), 20% fetal bovine serum (FBS) (Equitech Bio, Kerrville, TX), and 1% penicillin/streptomycin/fungizone (PSF, Sigma) until confluent in a 37° C, 5% CO_2, humidified environment.

Confluent cells of low passage from each isolation were released from culture plates by enzymatic treatment with 0.25% trypsin and seeded as micromass cultures on glass coverslips at a density of 4,000 cells/10µl for immunohistochemistry. Cells in the spot culture were allowed to attach to the substrate for one hour before the well was flooded with complete, serum-containing medium. Cultures were brought to quiescence by reducing the serum content from 10% to 2.5% over five days.

7.3.3 Tendon harvest and section preparation

Achilles tendons of right and left legs of male, wild type (*wt*), $P2Y_1$(-/-), and $P2Y_2$(-/-) mice were harvested using sterile techniques (n=2/group). Samples were immediately placed in a mold, placed on dry ice, and embedded in optimal cutting temperature (OCT) compound. Samples were stored at -80°C until sectioning was performed.

Saggital sections of each frozen tissue sample were made at a thickness of 10µm using a cryostat microtome. The resulting sections were placed on a labeled Fisherbrand Plus (Fischer Scientific, USA) microscope slide and stored at -80°C until immunohistochemistry was performed.

7.3.4 Staining Cx43, actin, and nuclei

Fluorescence microscopy of stained cells was used to determine the distribution of Cx43 in different cell types. Spot cultures and frozen tissue sections were rinsed two times with PBS and fixed in 3.7% formaldehyde for 30 minutes at room temperature. Samples were permeabilized with 0.2% Triton X-100 in 0.5%

bovine serum albumin for 30 minutes, and rinsed twice with PBS. Next, cells and frozen sections were treated with the primary antibody, rabbit anti-connexin-43 (Zymed Laboratories, San Francisco, CA) (1:1000 dilution), and allowed to incubate for 2 hours (cells) or overnight (frozen sections). Following three successive rinses with PBS, tissue sections were incubated in a goat, anti-rabbit, Alexa Fluor 488-labeled secondary antibody (1:250 dilution), 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes) (1:5000 dilution), and rhodamine phalloidin (1:1000; Molecular Probes, Eugene, OR) in the dark for 1 hour (cells) or 2 hours (frozen sections). Finally, sections were washed again two times with PBS to remove excess dye, mounted on cover slips, and stored at 4°C until fluorescence imaging analysis was performed. Specificity was verified by the absence of any staining in the negative controls (no addition of the primary antibody). Stained cells and frozen tissue sections were imaged with a fluorescence microscope (Olympus BX-60), equipped with an Olympus FV II digital camera and Olympus Microsuite B3SV image analysis software.

7.4 Results

Antibodies were used to fluorescently label Cx43 in tenocytes from *wt*, $P2Y_1(-/-)$, and $P2Y_2(-/-)$ mice. Cells from all groups displayed a complex and interconnected morphology within both the frozen tissue sections and monolayer cultures (Figures 7.1, 7.2). Cx43 proteins, immunolabeled and counterstained with Alexa 488, were visible as punctate localizations along the cell membrane (Figure 7.2). However, it appeared that the $P2Y_2(-/-)$ cells had fewer strands of punctate

spots, as compared to *wt* (Figures 7.2, 7.3). Moreover, $P2Y_2$(-/-) cells demonstrated a much higher prevalence of peri-nuclear and cytosolic Cx43 (Figure 7.2). This indicates a large amount of unassembled Cx43 monomer within the cell body. Phospho-connexin43 antibody detects endogenous levels of Cx43 when phosphorylated at serine 368 (Solan et al., 2003). This site is phosphorylated by PKC, and can decrease cell-cell communication.

7.5 Discussion

The involvement of gap junctions in the mechanotransduction response in tendon is well studied. (McNeilly et al., 1996; Banes et al., 2001; Wall & Banes 2005; Wall et al., 2007) However, this is the first report that purinoreceptors may modulate Cx43 expression. The purpose of this study was to elucidate Cx43 localizations within *wt* and purinoreceptor knockout mouse tenocytes.

Frozen sections from $P2Y_1$(-/-), $P2Y_2$(-/-), and *wt* mouse Achilles tendons, and cells from Achilles tendon tissues harvested from $P2Y_2$(-/-) and *wt* mice, were immunolabeled with Cx43 antibody in order to asses the presence and distribution of Cx43 within cells. Immunochemistry results reveal a decrease in Cx43 staining localization in $P2Y_2$(-/-) cells (Figure 7.2). Peri-nuclear staining was evident in the $P2Y_2$(-/-) cells, indicating a strong incidence of Cx43 monomers within the cytosol. The lack of assembled Cx43 proteins results in a decrease in the number of gap junctions joining cells. A single $P2Y_2$(-/-) cell responds to a mechanical poke, but fails to communicate an intercellular wave to neighboring cells (See Chapter 5). This indicates that cell signaling events may be linked to gap junction assembly,

phosphorylation, gating, or the act of embedding the formation into the plasma membrane.

Another noteworthy observation from immunolabeling experiments was the demonstration of robust actin filament staining within the $P2Y_2$(-/-) tenocytes (Figure 7.4). The density and thickness of stained actin filaments may translate into a more robust cytoskeleton and ultimately, a stiffer cell that is less able to withstand the rigors of excessive mechanical loading (Qi et al., 2006).

Intercellular signaling initiates a feedback response on cytoskeleton proteins, including actin (Watson, 1991; Wang, 2001). The inability of $P2Y_2$(-/-) tendon cells to transmit a load signal may result in a more resilient reorganization of the cytoskeleton within the stimulated cell. Scientists have proposed a mechanosensory tensegrity model to explain how cells dynamically adapt to their mechanical environment. In this rationalization, cells detect loading events through matrix-integrin interactions when forces are resisted by the ECM and deform the cell. Moreover, forces generated in the cytoskeleton are opposed by the ECM, causing another means of cell stress (Ingber, 2003).

The elasticity of a cell is influenced by the residual stress in its cytoskeleton, which itself is manipulated by matrix stiffness, attachment of the cell to the matrix, and cell-cell connections (Zhu et al., 2000; Chaudhuri et al., 2007). Researchers support the notion that residual stress in the cytoskeleton may provide a novel mechanical regulation of cellular biochemistry and mechanotransduction (Zhu et al., 2000; Hossain et al., 2006). Increased intracellular Ca^{2+} concentrations are the mainstream mechanism by which cells detect and respond to mechanical events

(Sanderson et al., 1994). A subsequent mobilization in IP_3 can navigate to neighboring cells through gap junctions to cause an intercellular calcium wave (von Kugelgen & Wetter, 2000). However, if gap junction expression is decreased, or if the assembled channels are in a closed (phosphorylated) state, this type of communication is not possible. The result would likely transpire into a stiffening of the cytoskeleton, as seen in the intracellular actin polymerization of $P2Y_2$(-/-) tenocytes.

There exists a clear association between purinoreceptors, Cx43, and actin. However, it remains unclear whether the altered signaling behavior caused the thickening of actin filaments and lack of gap junction formation, or whether the lack of communication in response to stress is the consequence of these physical malformations. Regardless, an important role of purinoreceptors has been identified in Cx43 localization and cytoskeletal configuration.

7.6 Acknowledgements

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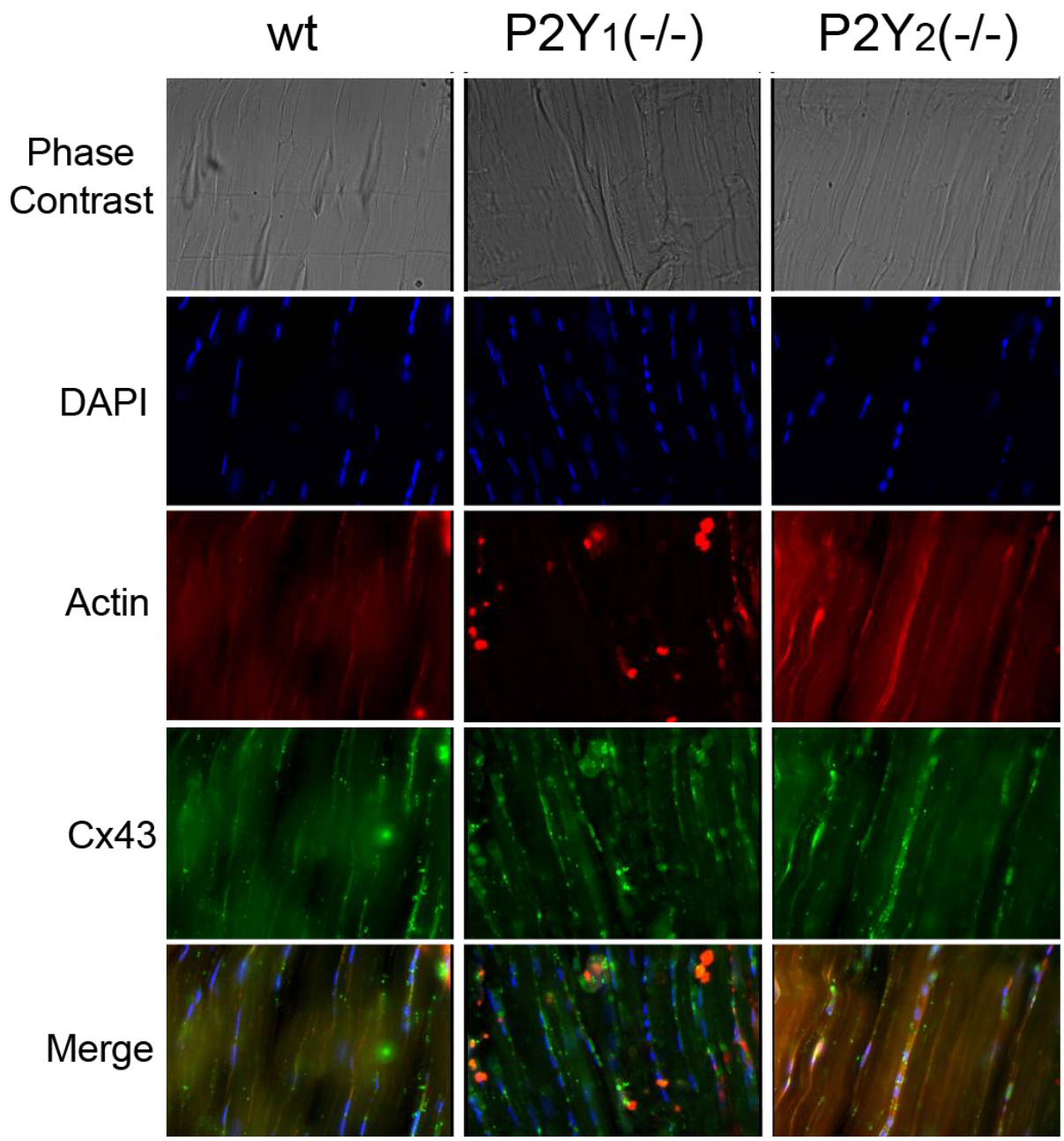

Figure 7.1 Images of *wt*, $P2Y_1(-/-)$, and $P2Y_2(-/-)$ frozen sections immunolabeled for Cx43 (green), actin (red), and DAPI-stained nuclei (blue).

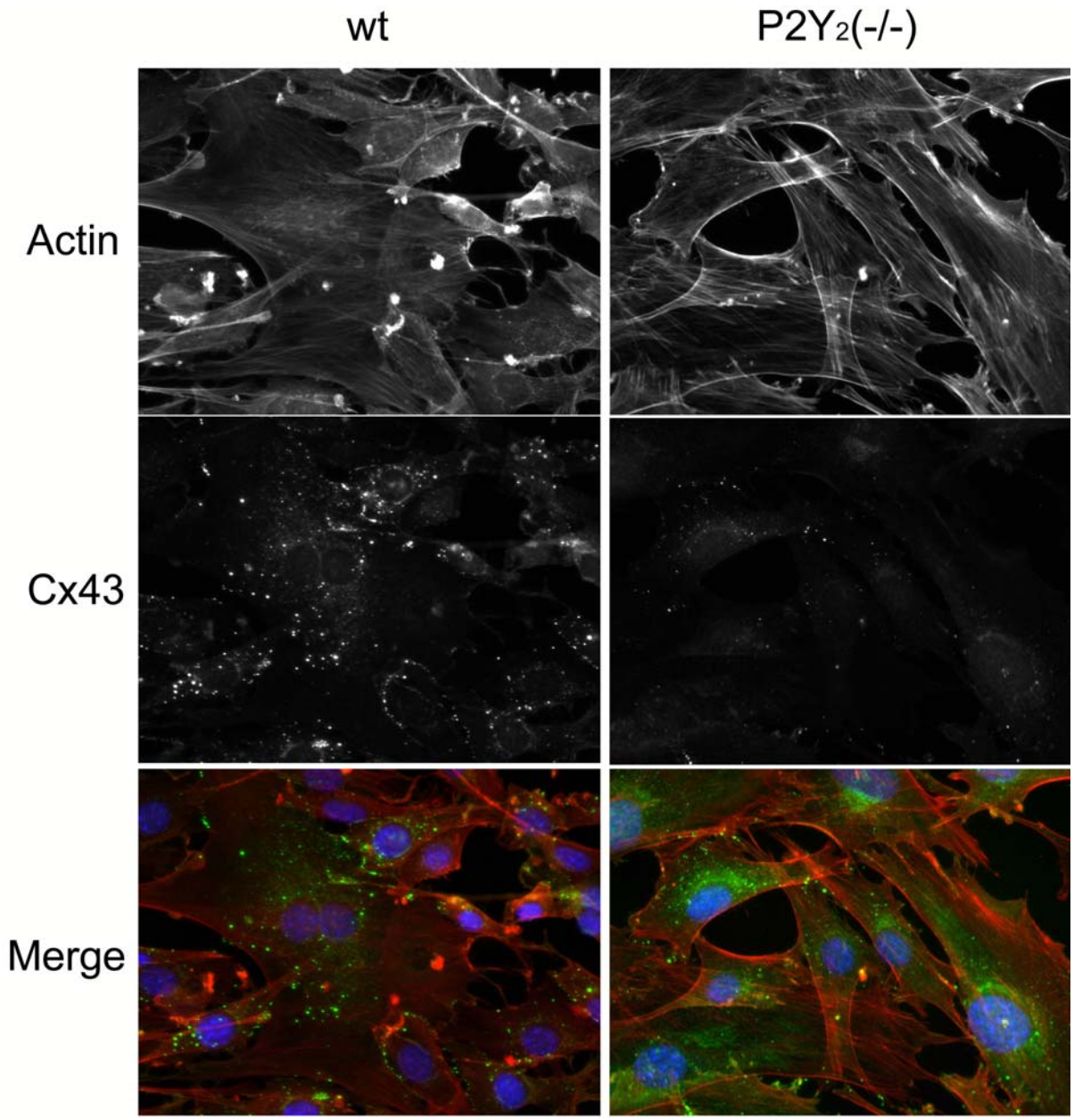

Figure 7.2 Representative images of *wt* and $P2Y_2$(-/-) tenocytes stained for Cx43 (green) and actin (red) (DAPI stained nuclei are blue). P2Y2(-/-) cells show some degree of punctate staining at points across the cell periphery, indicating Cx43 proteins are assembled into gap junctions. However, the amounts of cytosolic and peri-nuclear staining indicate a prevalence of Cx43 monomers or gap junctions that were not transported to the psuedopods of the cell.

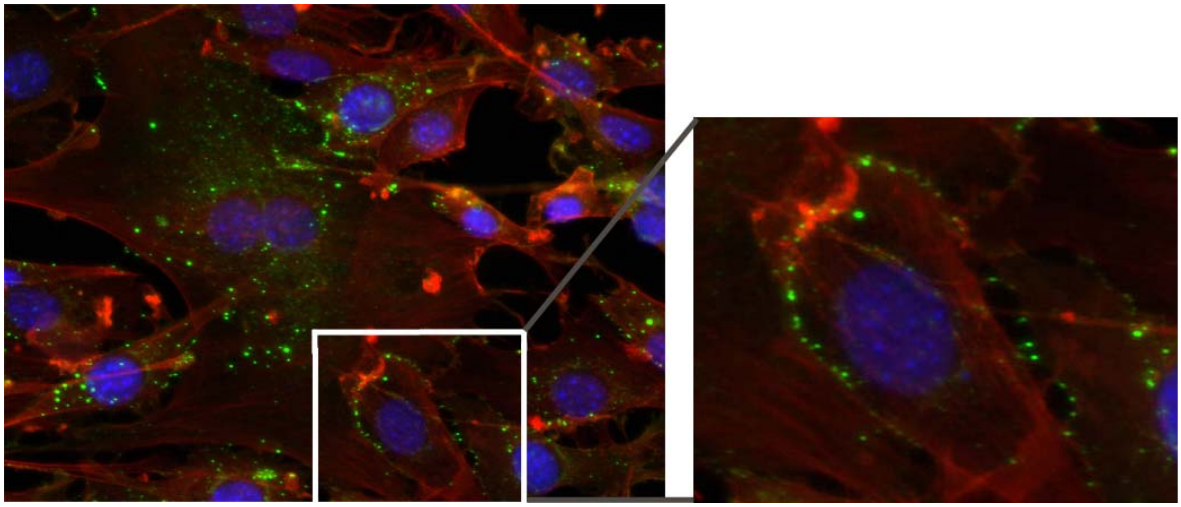

Figure 7.3 A magnified image of *wt* cells stained for Cx43 (green) and actin (red) (DAPI-stained nuclei are blue). Assembled Cx43 proteins form gap junctions along the periphery of *wt* cells.

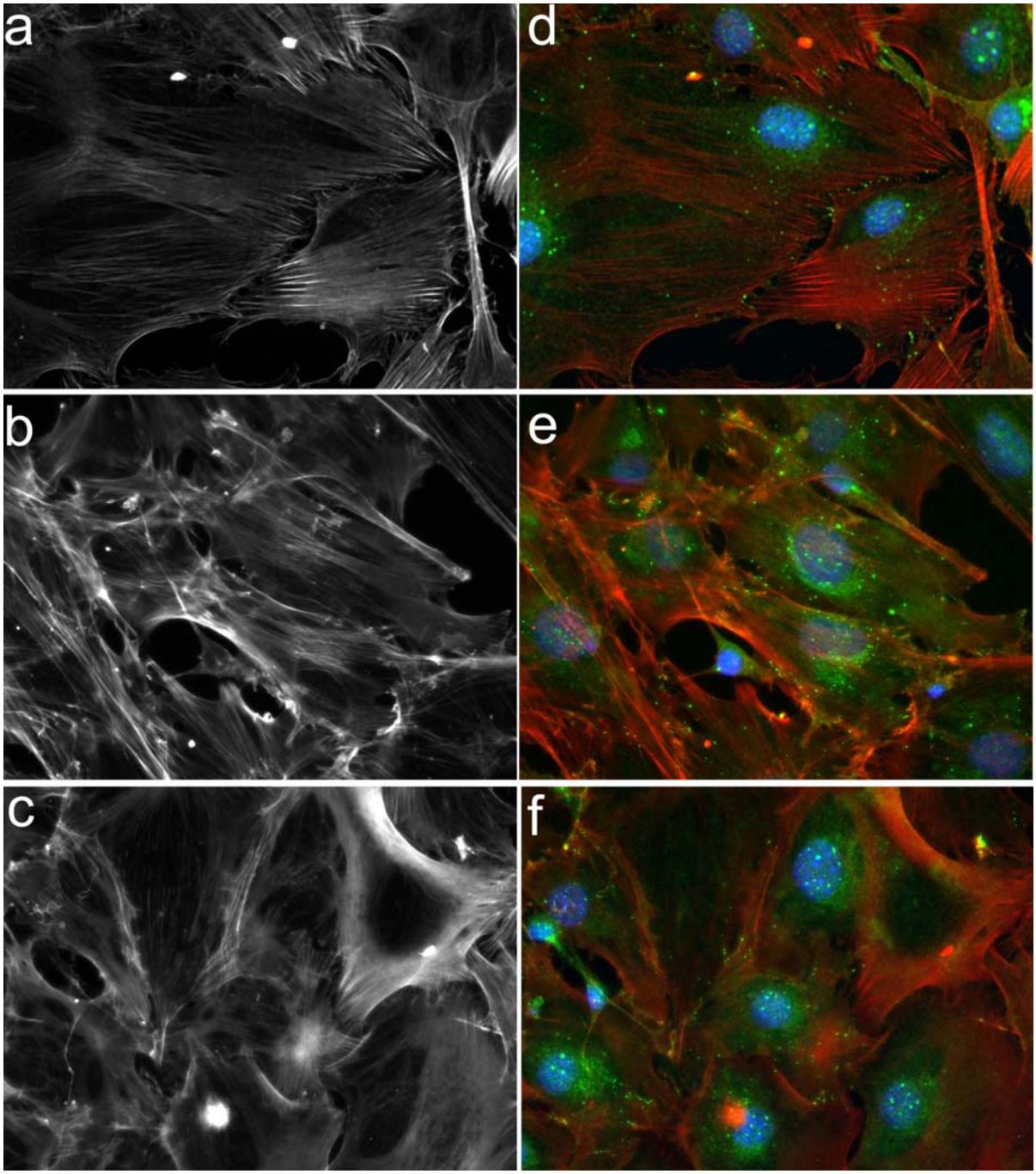

Figure 7.4 Robust staining of actin filaments (a-c) was evident in $P2Y_2$(-/-) tenocytes, indicating a cytoskeletal association with the decreased intercellular communication in response to mechanical stimulation. Figures d-f show a diffuse cytosolic staining, suggesting Cx43 monomer or unassembled gap junctions were present.

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8 Conclusions

8.1 Summary

ATP is used by cells as a diffusible messenger to incite the spread of intercellular calcium transients. Many cells including tendon release ATP in response to mechanical stimulation (Banes et al., 1995; Tsuzaki et al., 2003). This thesis has demonstrated $P2Y_2$ purinoreceptors are a necessary constituent of mechanotransduction of tendon cells, and without them cells are hindered in their ability to respond to load.

Calcium is a ubiquitous ion in all cells and has been shown to mediate a myriad of biological functions including fertilization, proliferation, and apoptosis (Sanderson et al., 1994). Mechanical stimulation of tenocytes results in an intercellular calcium communication mediated by gap junctions and purinoreceptors. Given that mechanical loads help maintain tendon homeostasis, and are often implicated in tendon injury, investigation of intercellular calcium signaling is an important paradigm for understanding cellular physiology. This is the first study to examine purinoreceptors in tenocyte intercellular calcium signaling. A series of real-time intercellular calcium imaging experiments with purinoreceptor null mice tenocytes enabled visualization and quantitation of responses following single-cell indentation and pharmacological agonist additions to cells. Data indicated that $P2Y_2$ receptors were associated with the increase in intracellular calcium, while ADP

acting through $P2Y_1$ receptors relieved the response. This negative feedback response is possible because of ecto-NTPases that are present on the tendon cell surface (Tsuzaki et al., 2003). Data presented in chapter 6 indicate that tenocytes lacking $P2Y_1$ and $P2Y_2$ purinoreceptors were able to elevate their calcium levels in response to a mechanical stimulus, indicating store-operated calcium channels were present and functional. However, the magnitude of communication to surrounding cells was markedly different. Cells lacking the ATP receptor failed to communicate a response to adjacent cells, suggesting a gap junction or second messenger deficit. Indeed, Cx43 immunohistochemistry uncovered a striking difference between the distribution of puncta in $P2Y_2$(-/-) versus *wt* cells. Instead of the typical 'string of pearls' staining associated with normal gap junction clusters between adjacent cells, $P2Y_2$(-/-) tenocytes displayed numerous internal puncta and a large amount of cx43 cytosolic staining. Results of immunochemical staining for cx43 in $P2Y_2$(-/-) cells indicate two possible explanations for the diffuse cytoplasmic staining and presence of internal puncta: 1.) previously functional gap junctions had been internalized and were being degraded, or 2.) cx43 monomers could not be transported from the Golgi apparatus to the plasma membrane where they could be assembled into functional gap junctions in an efficient manner. Diffuse staining within the cell points to an excess of Cx43 monomer, indicating a problem with trafficking and assembling connexin proteins into gap junctions. Results of other experiments in which $P2Y_2$(-/-) cells were subjected to fluid shear stress indicated that ATP release was reduced. Connexins have been identified as ATP-release channels (Bruzzone, 2001); therefore lack of functionally organized connexin 43 hemichannels is a potential

mechanism explaining why ATP release is reduced from $P2Y_2$(-/-) cells. However, $P2Y_2$(-/-) cells lacking the ability to communicate a response to a localized stimulus should respond to global stimuli such as those present during normal conditions *in vivo*. Therefore, these $P2Y_2$(-/-) cells provide a useful model for conditions within tendon that involve communication deficits. Moreover, microdamage that often precedes tendon pathology can occur in localized patterns, and the response behavior of $P2Y_2$(-/-) cells may represent a paradigm for this type of trauma.

$P2Y_1$(-/-) cells exhibited a hyper-communication phenomenon, with respect to levels of intracellular calcium ions, when a single cell was stimulated. The increased communication evident in these cells may lead to dangerously high levels of $[Ca^{2+}]_{ic}$ when these cells are exposed to a global stimulus, instead of a simple single-cell stimulation. Increased $[Ca^{2+}]_{ic}$ may activate pathways that block collagen 1 expression resulting in decreased strength of tendon, as well as changes in the microstructural morphology within the tissue. These points are under active investigation. *In vivo*, these hypersensitive $P2Y_1$(-/-) cells may respond to lower forces than *wt* cells, or may communicate a response to more cells when stimulated. In either situation, $P2Y_1$(-/-) cells may surpass a threshold present in *wt*, normal cells that keeps their resident tissues within a homeostatic balance. It is hypothesized that hypersignaling with $[Ca^{2+}]_{ic}$ could drive $P2Y_1$(-/-) cells to reduce collagen synthesis and/or activate a degradative pathway leading to matrix degeneration and ultimately, a weaker tissue. Therefore, the ability of a cell to moderate a response to load via $P2Y_1$ receptors may be required for normal tissue homeostasis.

The future of clinical treatments relies on a precise understanding of the molecular mechanisms that maintain normal tissue conditions, and how they may fail in pathogenic states. This body of work was focused primarily on the role of nucleotides in the mechanically-evoked calcium signaling response of tenocytes, the involvement of gap junctions in the response, and the downstream consequences on the structural integrity of the tissue. Information gathered from these studies indicates a potential animal model for tissue injury, as seen in the hyper-signaling response to mechanical loading as well as the downstream effects on matrix gene production and structural integrity of tendons in $P2Y_1$ purnioreceptor-null mice. Furthermore, ADP-based treatments or overexpression of $P2Y_1$ ADP-sensing receptors in deteriorating tissues may render a tissue more capable to modulate hyper-stimulation behavior that leads to tissue degradation. In contrast, $P2Y_2$ receptors may model communication deficient cells which may progress toward pathologic states.

8.2 Future work

An important modulatory role for $P2Y_1$ and $P2Y_2$ purinoreceptors has been identified in tenocyte response to load. However, the mechanism behind the communication disparities in knockout cells remains unknown. Several experiments are suggested below to elucidate potential origins of the signaling disparity.

In order to ascertain the functionality of gap junctions in *wt* and purinoreceptor knockout tenocytes, microinjection of dyes permeable through gap junctions could be investigated. This technique is capable of detecting if connexin hemichannels

are coupled, and could be further supported by TEM. Full PCR profiles could also elucidate the connexins present in mouse Achilles tendon, narrowing the focus. Furthermore, a problem may exist not in the connexins, but in other gap junctional proteins involved in the assembly, trafficking, docking, or phosphorylating events critical to its function.

Second messengers play an important role in cell-cell signaling. To elucidate whether the non-signaling $P2Y_2(-/-)$ tenocytes fail to produce a second messenger necessary for the communication of an intercellular calcium wave, a co-culture with live-cell staining could be used. *Wt* and $P2Y_2(-/-)$ tenocytes would be mixed at a 1:1 ratio and spot cultured onto a cover slip. The cells are allowed to attach to the substrate; the wells flooded with complete media, and allowed to proliferate to confluence. Following this technique, micropipette indentation of a wt cell (surrounded by ko cells) would release second messengers into $P2Y_2(-/-)$, and vice versa. If a normal signaling response is seen in $P2Y_2(-/-)$ cells, it would clearly be a second messenger quandary.

8.3 References

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