Cell Signaling in Tenocytes: Response to Load and Ligands in Health and Disease

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Abstract

Signaling in tenocytes during development, homeostasis and injury involves multiple and redundant pathways. Given that tendons transmit mechanical forces from muscle to bone to effect movement, a key function for tenocytes is the detection of and response to mechanical stimulation. Mechanotransduction involves matrix-integrin-cytoskeleton to nucleus signaling, gap junction intercellular communication, changes in intracellular calcium (Ca^{2+}), activation of receptors and their pathways, and responses to biochemical factors such as hormones, growth factors, adenosine triphosphate (ATP) and its derivatives, and neuromodulators. The primary cilium also plays a key role in the detection of mechanical signals. During development, transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), and hedgehog (Hh) signaling modulate tendon differentiation and formation. The response to injury is complex and varied involving not only inflammatory mediators such as interleukin-1β but also mechanosensing. This chapter reviews the signaling pathways tenocytes use during mechanotransduction, development and in response to injury.

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Introduction

Mechanical stimulation is important for maintaining tendon structure and function and may influence cellular responses to external factors, including hormones, growth factors, nucleotides, and neurotransmitters, possibly released from surrounding blood vessels and nerves during trauma to the tendon (Fig. 7.1 [13, 49]). Specific signaling pathways may be activated in response to mechanical stimulation that drive matrigenesis, mitogenesis (MEK/MAPK), a stress response (JAK/STAT and JNK/SAPK), apoptosis, or other responses [4, 7, 11, 14, 15, 62–64]. The extent of the response can be altered by the microenvironment itself. Tenocytes have a homeostatic set point provided by cytoskeletal tension and connections to the extracellular matrix, that when altered, changes tenocyte shape, increases matrix metalloproteinase-13 (MMP-13) mRNA expression, and alters cilia length [5, 44]. Tenocyte responses to both physical and chemical changes in their environment may involve second messengers (such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate, ATP, guanosine triphosphate, nitric oxide, prostaglandin E2 (PGE_2), inositol triphosphate (IP_3), and diacylglycerol) that can act in autocrine and/or paracrine fashions, ion channels (such as Ca^{2+}-dependent, Ca^{2+}-independent, stretch-activated and voltage-gated), changes in RNA and protein expression, cilia deformation, cytoskeletal interactions (involving integrins, focal adhesion kinase, paxillin, filamin, integrin-linked kinase, vinculin, and talin), changes in intracellular calcium concentration ($[Ca^{2+}]_{ic}$), and intercellular communication, among other mechanisms (Figs. 7.1 and 7.4).

The detection of mechanical signals is key for tendon function given that their primary role is to transmit mechanical forces from muscle to bone to effect movement. Under normal physiologic conditions, tendons are subjected to low strains. At 1 % strain in whole tendon *in vivo*, the matrix crimp pattern is unaffected and cells are nominally deformed [6]. However, an applied 3 % strain deforms collagen fibrils to a straightened position. The tendon is taut and can undergo reversible deformation thus, the cells are subjected to a level of deformation above a nominal threshold. At 5 % strain, the tendon is subjected to the upper limit of elastic deformation and suffers some plastic deformation. Plastic deformation could include cell-cell contact disruption or alterations in protein arrangement within the tenocyte plasma membrane, such as connexin 43 (Cx43), the primary constituent of gap junction channels in tenocytes [72] and initiate an inflammatory response that involves interleukins, MMPs, and insulin like growth factor [88, 89, 93, 95].

This chapter will review some of the key mechanisms tenocytes use in mechanotransduction, including Ca^{2+} signaling, intercellular communication via gap junctions, norepinephrine (NE) activation of adrenoceptors, ATP and purinoceptors, and primary cilium. In addition, this chapter will review signaling pathways used during tendon development and in response to injury.

Tenocyte Mechanotransduction

Calcium Signaling and Gap Junction Intercellular Communication

One method by which tenocytes can detect and respond to mechanical stimulation is through intercellular communication pathways whereby $[Ca^{2+}]_{ic}$ increases in a coordinated fashion among interconnected cells [17]. Calcium wave propagation has been demonstrated in many cell types and can occur through direct intercellular signaling via gap junctions [25, 87], paracrine signaling mechanisms [32, 56, 57], or both [28, 56, 57]. Cells can propagate Ca^{2+} waves to neighboring cells through the passage of IP_3, or a signal that produces IP_3 in neighboring cells, through gap junctions and activation of IP_3 receptors on the endoplasmic reticulum [17]. However, it has become apparent that cells can use multiple mechanisms to propagate intercellular Ca^{2+} waves, including the release of nucleotides (e.g., ATP, uridine triphosphate (UTP)) and

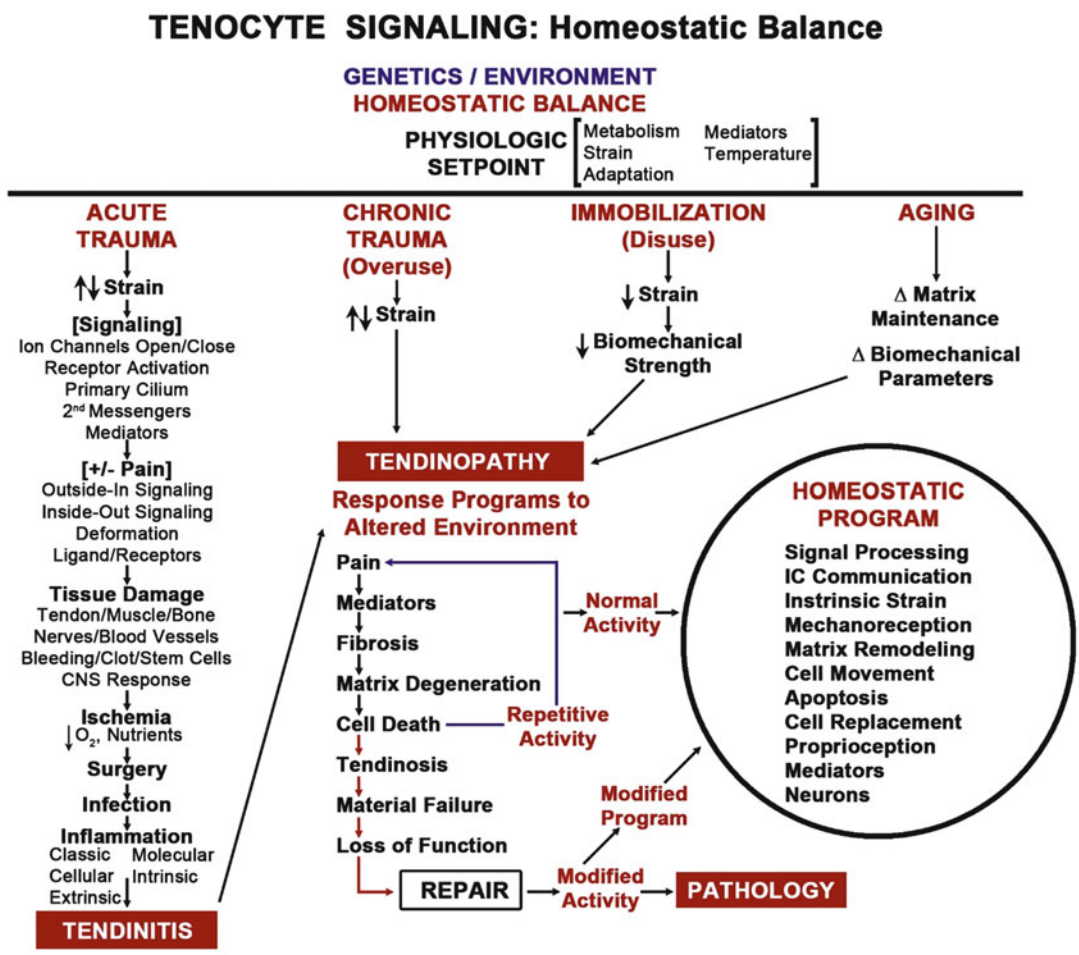

Fig. 7.1 A synopsis of normal and pathologic processes that results in homeostatic balance or pathologic process in tenocyte signaling pathways (Altered with permission from Banes et al. [13])

subsequent activation of purinoceptors on neighboring cells [28, 55–57].

Tenocytes *in vivo* have immunohistochemically detectable gap junctions [72]. *In vitro*, human tenocytes form functional gap junctions in both monolayer and three-dimensional collagen gels [60]. Gap junction channels form between cells when hexameric gap junction protein (connexins) structures on neighboring cells dock to create a pathway for direct intercellular exchange of ions and molecules [39, 40, 86, 107]. Connexins have been shown to be associated with the actin cytoskeleton, which may help stabilize gap junctions during periods of prolonged mechanical loading [103]. Additionally, connexin hemichannels can contribute to intercellular communication via autocrine and paracrine pathways (see [47] for review). *In vitro*, Cx32 and Cx43 are expressed in human, avian, murine and equine tenocytes, with Cx43 being the most prevalent species [17, 60, 107]. Avian tenocytes also express Cx26 [17]. Cx43 connects tenocytes in a syncytium, whereas Cx32 connects tenocytes between syncytial layers (Fig. 7.2 [72, 107]).

In tendon, gap junctions are involved in mechanotransduction pathways [101]. The magnitude of the applied mechanical load can alter gap junction intercellular communication in tenocytes, where low levels of strain (4 %)

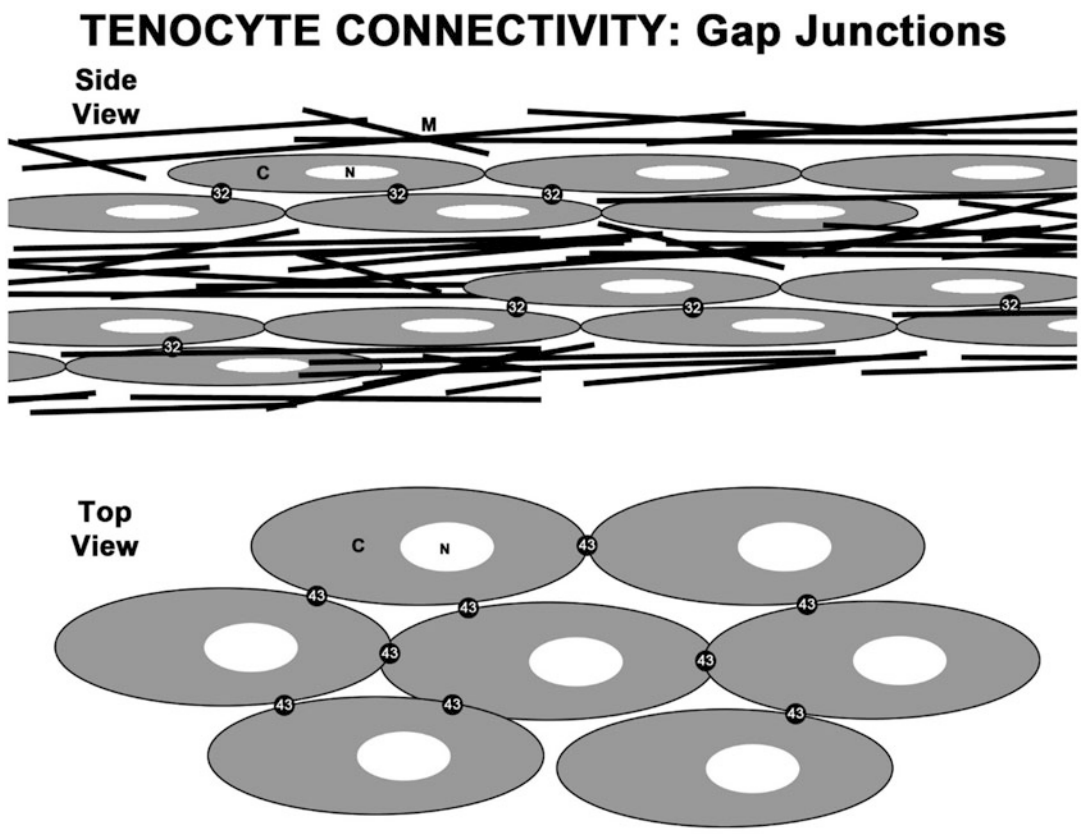

Fig. 7.2 *Side view* is a view of multiple tenocyte syncytial layers connected by connexin (Cx) 32; *Top view* is an in face view of a single tenocyte syncytial layer connected by Cx43. 32, Cx32 gap junctions connecting over and underlying tenocyte syncytia; 43, Cx43 gap junctions in adjacent cells in a syncytium; *C* cell body, *N* nucleus, *M* extracellular matrix

increased communication but high levels of strain (8 %) decreased communication [69]. Gap junctions also modulate load-induced DNA and collagen synthesis, which may contribute to matrix remodeling in response to load [12]. Results of a study by Waggett et al. [100] indicated that signaling mediated by Cx43 gap junctions may inhibit load-induced collagen secretion, whereas Cx32 signaling may stimulate load-induced collagen secretion in tenocytes. Thus, a change in connexin expression or signaling could significantly alter the molecular signals transferred between cells [46] and potentially the physiological response of the tenocyte.

Gap junctions are gated by several mechanisms including phosphorylation of serine residues on connexins. The full promoter sequence for Cx43 contains two activator protein-1 (AP-1) and four cAMP response elements (CRE)-like sites that are reported to mediate cellular response to cAMP [108]. As a second messenger, cAMP can modulate changes in gene expression via activation of transcription factors (e.g., CREB) that bind to specific promoter sequences that drive gene transcription. In avian tenocytes, cyclic equibiaxial strain activated cAMP response element binding (CREB) protein and AP-1 transcription factors [16]. Cyclic AMP as well as Ca^{2+} can increase protein kinase A and C activation, respectively, which ultimately results in Cx43 phosphorylation [27, 86]. Norepinephrine activation of adrenoceptors can also increase cAMP and $[Ca^{2+}]_{ic}$ in connective tissues [21, 59, 102]. Thus,

strain and NE may activate common or additive site(s) in the Cx43 promoter to drive transcription. Other ligands may also act to alter Cx43 expression during injury or disease. IL-1β was found to upregulate Cx43 expression in tenocytes [83]. Furthermore, strain-induced cell death is gap junction-dependent [83].

Norepinephrine and Adrenoceptors

Norepinephrine is a neurotransmitter that binds adrenoceptors, G-protein coupled receptors important in the regulation of many functions including vasoconstriction (see Chap. 4) [34, 48, 99]. Cells from avian tendons express the α_{1A} and α_{1B}-adrenoreceptor subtypes [102] and respond to NE by increasing $[Ca^{2+}]_{ic}$ primarily via activation of α_{1A}-adrenoreceptors [102]. Human and rabbit tenocytes have been found to express α_{2A}-adrenoceptors [8]. Normal human tendons show positive immunoreactivity for adrenoceptors in both the tendon proper and in the tendon blood vessel walls [33]. Human tenocytes also express tyrosine hydroxylase which suggests that tenocytes may be capable of producing endogenous catecholamines [8]. Additionally, tendons that present with tendinosis have increased immunoreactivity for α_1-adrenoreceptors in the blood vessel walls suggesting a role of catecholamines in tendinopathy [33]. Thus, adrenoceptors may provide a mechanism for neurohormonal modulation of tenocyte function under both normal and diseased states.

Purinoceptors and ATP

Purinoceptors are metabotropic, G protein coupled (P2Y class) or ionotropic, ligand-gated ion channels (P2X class; [31]). $P2Y_2$ reacts with ATP or UTP, and $P2Y_1$ reacts primarily with adenosine diphosphate (ADP) but partially with ATP [30, 31]. In tendon, ATP activates $P2Y_2$ purinoceptors [97]. Tenocytes secrete ATP particularly in response to fluid shear or stretch [97]. 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 [96]. Addition of 1 μM ATP to tenocytes or ligament cells *in vitro* and in whole tendons *ex vivo* increased $[Ca^{2+}]_{ic}$ [44, 55]. ATP and UTP may also act to amplify responses to mechanical stimulation because they activate a common pathway through an increase in $[Ca^{2+}]_{ic}$ [97]. Tenocytes from $P2Y_2$ knockout mice that lack the receptor for ATP do not respond to substrate strain by increasing $[Ca^{2+}]_{ic}$ [54]. ATP can also modulate the contraction of a linear, three-dimensional, collagen gel seeded with tenocytes or MC3T3-E1 cells [84] indicating a role of ATP in matrix remodeling. Increased ATP secretion may activate an inhibitory pathway, which may dampen a response to load [96, 97]. Additionally, ATP or a breakdown product, such as ADP or adenosine, may act as a stop, or modulating signal(s) for some genes impacted by mechanical load.

In addition to modulating responses to mechanical load, ATP is an important modulator of inflammatory gene expression in tenocytes. ATP can inhibit IL-1β-induced MMP mRNA and protein expression, cyclooxygenase-2 (COX2) expression, and PGE_2 secretion [97].

Deformation Sensing

Tenocytes can detect mechanical signals and, in turn, impose a mechanical signal via the matrix and/or substrate upon which they are cultured through both autobaric and parabaric effects (Fig. 7.3). Tenocytes detect substrate strain [104] likely via integrin connections to matrix through tensegrity [51]. More recently, tenocytes have been shown to also utilize the primary cilium to detect strain [61]. The illustration in Fig. 7.3 depicts how tenocytes respond to substrate strain, individually and in a syncytium. In the case of an autobaric effect, a tenocyte may respond to strain or a ligand by contracting, hence loading the cell itself and the matrix to which it is attached. The cell may then spread and flatten, altering its substrate even further. In a second set of responses, a tenocyte may alter

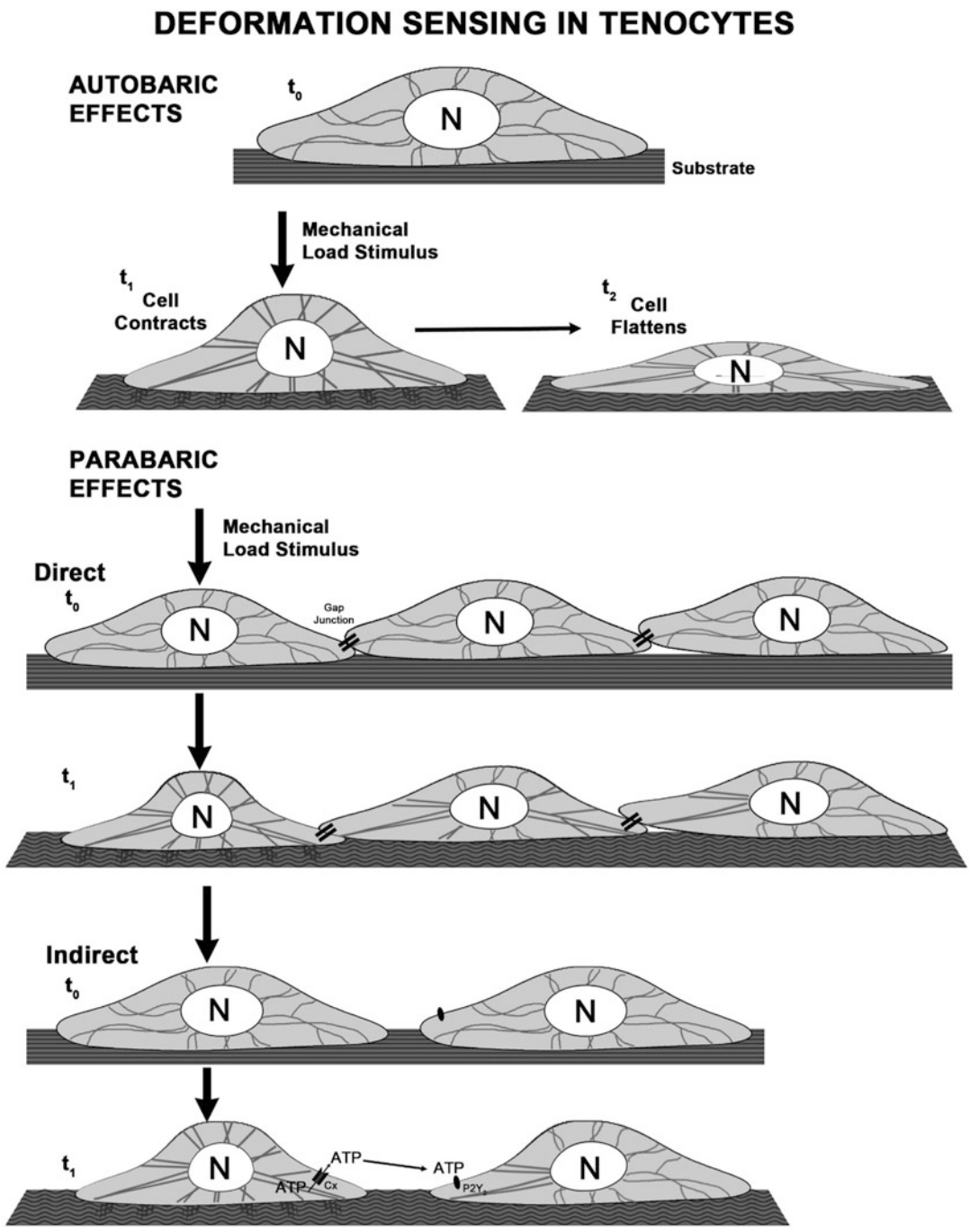

Fig. 7.3 Autobaric effects – At t_0, a cell receives a mechanical signal by deformation, and the response is a cell contraction event. The cell contracts and flattens (t_1) thus applying a secondary deformation to itself. Parabaric effects – Similar to the autobaric response to a mechanical stimulus, a cell or series of cells receive a signal then applies deformation via contraction to themselves and adjacent cells. The cell-driven deformation can be direct when cells are directly attached or indirect if cells are adjacent and deform the matrix, thereby transmitting load to an adjacent cell. In addition, in direct effects, the signal can be transmitted to adjacent cells via gap junction intercellular communication. In indirect effects, the signal can also be transmitted to neighboring cells via second messengers such as the release of adenosine triphosphate (ATP) through connexin (Cx) hemichannels from the mechanically loaded cell. The ATP can then activate purinoceptors ($P2Y_2$) on neighboring non-stimulated cells. (Altered with permission from Banes et al. [13])

shape by flattening, spreading and if attached to other tenocytes in a syncytium, will also apply load to attached cells in a direct parabaric effect. If a responding tenocyte is mechanically loaded but not directly attached to a neighboring cell, it may apply its load to the substrate and indirectly to the neighboring tenocyte in an indirect parabaric effect. In each case, the tenocyte can signal to cells via gap junctions or purinoceptors.

Role of Primary Cilia in Tenocytes

Primary cilia were discovered on mammalian cells in 1898, though they have been largely regarded as vestigial organelles for a majority of that time since their initial discovery [109]. Research over the last 20 years has revealed that primary cilia are prevalent on nearly all somatic cells. A typical (non-motile) primary cilium is composed of: (1) a tubular axoneme, approximately 0.2 microns in diameter and up to 10 microns in length, delimited by the ciliary membrane (contiguous with the cell's plasma membrane) and typically projecting outward from the cell body, (2) the axoneme containing nine circumferential microtubule doublets, devoid of a central pair of microtubules arranged in a 9 + 0 configuration, (3) a cylindrical basal body, of nine microtubular triplets derived from the mother centriole, which nucleate the microtubules of the axoneme [92], and (4) transition fibers and a basal foot that anchor the primary cilium to the cell membrane and/or ciliary pocket and the actin cytoskeleton [73]. The cilium is typically observed in a juxtanuclear position and often colocalizes with the Golgi apparatus [78]. The expression of the primary cilium is intimately associated with the phases of the cell cycle; however, there is some evidence that the details of this characteristic may be cell-type specific. Tenocytes *in vivo* and *in vitro* have primary cilium that face each other and vary in length [85].

Primary cilia generally present characteristics of chemo and mechanosensitivity and are thought to, in part, coordinate mechanotransduction pathways, particularly in mechano-active connective tissues [74, 75, 105]. Additionally, a variety of important signaling pathways localize their signaling activity to the base and axoneme of the primary cilium, including proteins of the Hh, Wnt, TGF-β, and platelet-derived growth factor pathways [81]. Though several important signaling mechanisms localize their activity to the primary cilium, many of the underlying mechanisms behind cilia expression and function remain elusive.

In the context of connective tissue, primary cilia were initially observed in cartilage [78, 79] and bone [105]. Imaging primary cilia in dense 3-dimensional (3D) connective tissue for quantitative analysis requires a sophisticated approach to generate accurate data on the length, shape and dimensions of the primary cilium. Groups have analyzed ciliary structure with transmission electron microscopy, epi-fluorescence microscopy with deconvolution, standard confocal fluorescence microscopy and multi-photon microscopy. Farnum and coworkers developed a method of morphometric analysis through using mulitphoton microscopy in such a way that allows them breakdown planar ciliary angle as well as elevation angle. Additionally, their approach allowed them to identify the prominence of primary cilium in the dense extracellular matrix of tendon and cartilage tissue [1, 41]. Farnum and Wilsman [42] further pioneered a more extensive analysis of the primary cilia in dense connective tissue, such as cartilage and tendon, to generate a more thorough understanding of the relationship between cilia orientation and mechanosensitivity.

Ciliary mechanosensitivity has been largely demonstrated in changes in cilia-associated proteins in response to fluid shear stress. These studies empirically demonstrated that primary cilia mediate mechanotransduction of shear forces in osteoblast cell types [70]. Other work in cartilage has shown that healthy and diseased cartilage tissue differentially express primary cilia, likely dependent on the mechanical environment that develops during osteoarthritis. Following these studies, cilia mechanosensitivity has been demonstrated in tendon explants cultured under cyclic tensile strain

[45, 61]. Gardner et al. [45] demonstrated that stress deprivation of tendon explants induced cilia elongation in tenocytes within 24 h, whereas cyclic tensile loading at 3 % strain induced a shortening of ciliary length. The authors suggest that in the absence of loading typically experienced by tendon *in situ*, tenocytes extend their cilia to increase their sensitivity to detect mechanical changes in their environment. In the study by Lavagnino et al. [61], rat tail tendon fascicles were incrementally exposed to strain levels up to 8 % and the ciliary deflection angle was measured at each strain increment. Detection of changes in ciliary deflection under this range of strains indicated scaling of deflection concordant with physiologic loading *in vivo*, supporting the hypothesis that cilia play a role in tendon mechanosensory mechanisms [61].

Other studies in mesenchymal (MSCs) and adipose stem cells (ASCs) have shown that the primary cilium may play a role in lineage specification [24, 50, 98]. Disrupting the primary cilium structure and/or specific cilia associated proteins results in downregulation of gene expression and end-product markers of osteogenic, chondrogenic and adipogenic differentiation [24, 50, 98]. Further, evidence suggests that culture under chemical induction towards osteogenic, chondrogenic and adipogenic lineages confers differential cilia expression linked to lineage specification. Cell morphology is a cursory indicator for cell phenotype and there is evidence that the primary cilium's length and orientation is in part modulated by cell shape and thus cytoskeletal organization. McMurray et al. [71] reported that when MSCs were cultured on grooved substrates, MSCs tended to orient along the direction of the grooves yielding an elongated cell morphology as well as elongated cilia which oriented with the long axis of the cell. This study also reported modulations of ciliary localized Wnt signaling in response to changes in culture substrate architecture. Similar observations have been reported on tenocytes in rat extensor tendons, which orient their cell body and cilia along the direction of the collagen fibrils and the long axis of the tendon tissue [35]. It follows that this principle likely extends to other connective tissue cells with mechanosensitive cilia, including tenocytes and that cilia expression may be involved in healthy and or disease tenocyte physiology. These data taken together suggest that the mechanosensitivity of primary cilia is, in part, indicative of the physiological state of connective tissue cell types and may also be involved in maintenance of tenocytes and/or tendon tissue physiology (Fig. 7.4).

Signaling in Tendon Development

The embryological origin of tendons is dependent on their anatomical position: axial tendons are derived from cells at the interface of the sclerotome and myotome known as the syndetome, limb tendons are derived from the lateral plate mesoderm, and cranial tendons, like other cranial mesenchyme, are neural crest derived. These embryological origins were clearly defined following the discovery of the basic helix-loop-helix transcription factor scleraxis (Scx), which is one of the earliest markers that specifies tendon primordia [91]. The progenitor cell populations that contribute to the initiation and differentiation of embryonic tendon are becoming clearer. Fate mapping studies indicate that two distinct progenitor pools exist at the time of tendon condensation: one pool that gives rise to the midsubstance and another pool that gives rise to the enthesis (i.e., tendon-to-bone insertion site) [22, 36, 90, 94]. While our understanding is not complete, there are known signaling pathways and transcription factors that regulate the specification and differentiation of these progenitor pools into their respective regions.

Around the time of condensation, the progenitor pool that gives rise to the enthesis expresses both Scx and the SRY-related transcription factor Sox9, while unlike the Scx-only population of the midsubstance and Sox9-only population of the underlying boney eminence [22]. Enthesis cells can also be traced back to a Gli1 and Gdf5 origin unlike the tendon midsubstance [36, 90]. Tight regulation of TGF-β and BMP

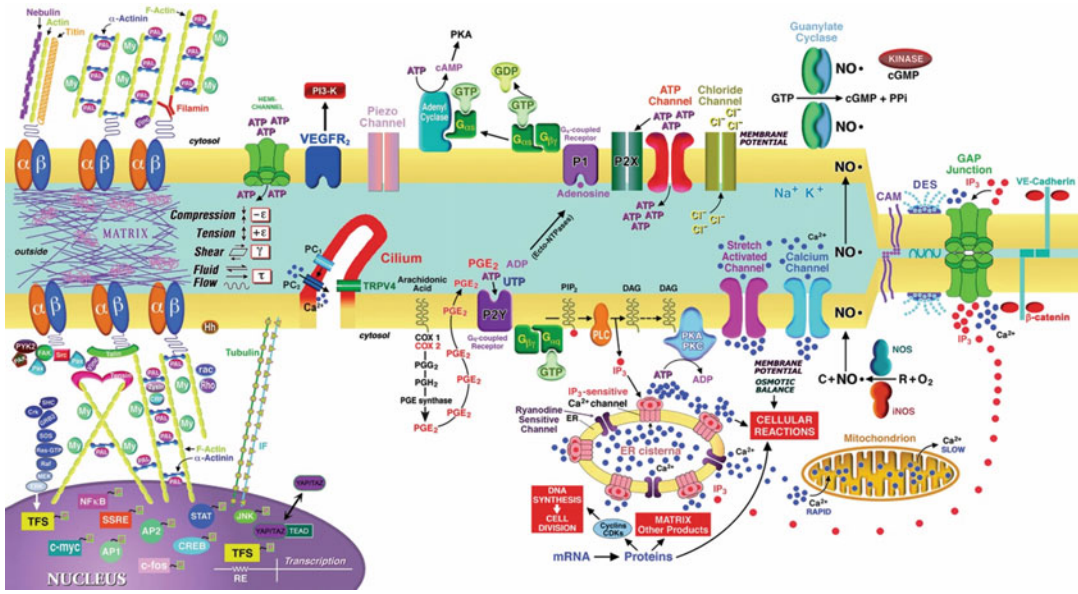

Fig. 7.4 The detection of and response to external mechanical stimuli (i.e., compression, tension, shear, fluid flow) involves multiple pathways and signaling mediators. A matrix-integrin-mechanosensory protein complex-cytoskeleton machinery is linked to a kinase cascade (tyrosine or nontyrosine kinase cascade or the JACSTAT kinase cascade) system. A mechanosensory protein complex contains talin, vinculin (Vinc), tensin, paxillin (PAX), Src, and focal adhesion kinase (FAK). In this model, a load deformation displaces matrix molecules tethered to clustered integrins at focal adhesions. The displacement is transduced to an integrin (β), to an integrin-binding protein, and then to associated proteins. Matrix-integrin-cytoskeletal interactions may also involve actin, myosin (My), nebulin, titin, α-actinin, filamin, palladin (PAL), tublin, and intermediate filaments (IF). Activated extracellular signal-regulated protein kinases (ERK) enter the nucleus and up-regulate transcription factor expression (TFS, AP1, AP2, SSRE, CREB, c-fos, c-myc,STAT, JNK) and activate nuclear binding proteins, such as nuclear factor κB (NF-κB; P for phosphorylation). Polycistin-1 (PC_1) is co-localized with the primary cilium and activated when the cilium is deformed by fluid shear stress. The shear stress signal is transferred from PC_1 to polycistin-2 (PC_2) and induces the influx of calcium (Ca^{2+}) though PC_2, which in turn activates ryanodine receptors in the endoplasmic reticulum (ER) to release Ca^{2+}, resulting in Ca^{2+}–induced Ca^{2+} release. Changes in intracellular Ca^{2+} through the release of intracellular Ca^{2+} stores from the ER through or entry of extracellular Ca^{2+} through channels such as the store-operated, stretch-activated, mechanosensitive Ca^{2+} channels, and voltage independent or dependent Ca^{2+} channels. The release of adenosine triphosphate (ATP) and, at lower levels, uridine triphosphate (UTP), following the activation of ionotropic P2X and metabotropic, G protein-coupled P2Y receptors in an autocrine/paracrine fashion. ATP acts on $P2Y_2$ receptors, the primary ATP/UTP responsive receptor in tenocytes, activating the Gαq-protein, driving phospholipase C (PLC) and producing inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on IP_3-sensitive Ca^{2+} channels in the ER to mobilize intracellular Ca^{2+}, and DAG activates a protein kinase C (PKC) pathway. Activation of adenyl cyclase activity yields cyclic adenosine monophosphate (cAMP), which stimulates cAMP-dependent protein kinase A (PKA), which may act at Raf in the kinase cascade. Initial action of ATP is terminated quickly by membrane-bound ecto-NTPases to its metabolites: ADP, AMP, and adenosine. Adenosine activates G protein-coupled P1 receptors, activating stimulatory (Gs) or inhibitory (Gi) signaling. Phosphoinositide 3-kinase (PI_3K) are activated by vascular endothelial growth factor receptor ($VEGFR_2$). Gap junctions pass IP_3, which propagates a Ca^{2+} wave from cell to cell after a mechanical signal is detected. Connexin hemichannels can pass ATP outside the cell. CAM, cell adhesion molecule; DES, desmosome; PPi, pyrophosphate; AP-1, activator protein-1; AP-2, activator protein-2; CREB, cAMP response element binding protein; MEK, MAPK/ERK kinase; NO, nitric oxide; PKB, protein kinase B; STAT, signal transducer and activator of transcription; SHC, Src homology protein complex; Crk, Src homology adaptor protein that binds paxillin and C3G; GRB_2, growth factor receptor binding adaptor protein linking receptors to the Ras pathway through FAK and SOS (Son of Sevenless), a guanine nucleotide exchange factor; Ras, GTPase that regulates activation of Raf; IF, intermediate filament; YAP/ TAZ, Yki transcription co-activators; TEAD, transcription factor; PYK2, a nonreceptor tyrosine kinase of the FAK family; PAK, p21-actived kinase; SSRE, shear stress response element; JNK, c-Jun N-terminal kinase; Hh, hedgehog; TRPV4, transient receptor potential vanilloid 4 channel; COX 1, cyclooxygenase 1; COX 2, cyclooxygenase. (Used with permission from Flexcell International Corp.)

signaling within this region controls the specification and differentiation of these progenitor pools [18, 22, 23, 82]. TGF-β signaling is crucial for the specification of the boney eminence and is also critical for the formation and differentiation of the tendon midsubstance [9, 22, 82]. BMP4 signaling also regulates the cartilage differentiation of bone eminence progenitors [22, 23]. Following the initial specification and differentiation of the enthesis progenitors, Hh signaling becomes prominent (Fig. 7.4 [68, 90]). Overexpression of Hh signaling in Scx-expressing progenitors yields production of enthesis extracellular matrix components within the midsubstance [68], suggesting that Hh signaling is important in the maturation process from enthesis progenitors to fibrocartilage cells. Hh signaling also regulates the mineralization of enthesis cells from unmineralized fibrochondrocytes to mineralized fibrochondrocytes [26, 36, 90]. Conditional deletion of Hh signaling in these cells leads to a severe reduction in mineralized fibrocartilage production. Therefore, Hh signaling is critical to the formation of the mineralized fibrocartilage zone of the enthesis.

The specification of the midsubstance progenitor pool is regulated via TGF-β signaling as removal of TGF-β signaling in limb mesenchyme leads to complete loss of limb tendon formation [82]. While the role of TGF-β signaling in the later differentiation and maturation events is less clear because a lack of inducible knockout models, it is likely that TGF-β signaling continues to play a prominent role because of its function in collagen transcription. TGF-β signaling has been shown to stimulate collagen transcription in a variety of cell types including tendon fibroblasts [19, 29, 66]. It stimulates the expression of both Scx and the Mohawk (Mkx) homeobox transcription factor, another factor important in tenogenesis, via Smad3 interactions [19]. In fact, Scx has known binding sites to both Cola1 and Col1a2 promoters [10, 66]. Therefore, TGF-β-mediated regulation of these transcription factors likely plays a role in collagen transcription during tendon development.

While the majority of *in vivo* cell signaling data related to tenogenic differentiation has come from embryological studies, less is known about the cell signaling events that regulate cell turnover during growth and tissue maintenance. In order to better understand the signaling events that regulate tenocyte turnover during these periods, *in vivo* models are needed as maintaining cell phenotype within *in vitro* systems is difficult [64]. While recent studies indicate that resident progenitors exist within tendon tissue and these cells can be isolated, expanded in culture, and display *in vitro* multipotent potential [20, 76], fewer studies have demonstrated expansion and differentiation of resident progenitors in an *in vivo* system. Using an alpha smooth muscle actin Cre reporter system (αSMA-CreERT2; R26R-tdTomato), Dyment et al. [37] demonstrated that SMA-labeled cells within the paratenon and/or perivasculature contribute to ScxGFP+ cells during tendon healing. This model system also labels a proliferative internal population within growing tendon [37] that may be a resident progenitor population. *In vivo* fate mapping models such as these are needed to characterize resident progenitor populations as they give rise to mature tenocytes. Unfortunately, improved markers are needed to better classify cells as they progress through the lineage. Hopefully with improved transgenic models and sensitive techniques such as single cell analyses, the markers that define cells at multiple stages of the lineage and the signaling pathways that regulate this process will be elucidated in the not so distant future.

Inflammation and Response to Injury

Inflammation in response to injury is complex, which is no less true in the tendon than in any other tissue (see Chap. 20). Tenocytes show an expected variety of reactions to inflammatory signals typically associated with the activation of resident immune cells and the recruitment of inflammatory cells. However, inflammation in tendon is complicated by the fact that injuries often take the form of mechanical injuries to the extracellular matrix. Therefore, the inflammatory

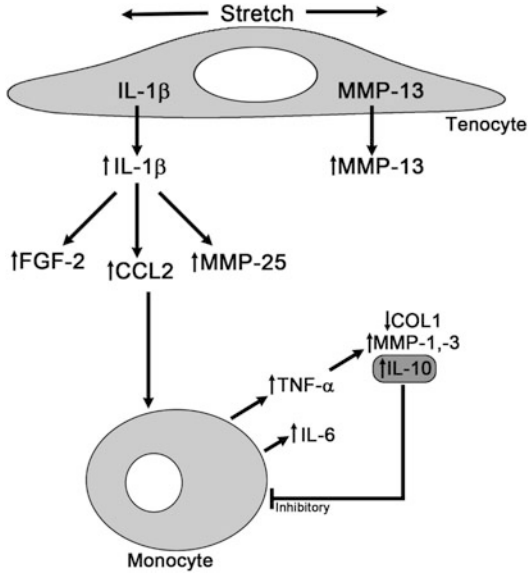

Fig. 7.5 Stretch induces tenocytes to produce interleukin-1β (IL-1β) and matrix metalloproteinase-13 (MMP-13). IL-1β can act in an autocrine and paracrine fashion to generate repair signals (such as fibroblast growth factor-2 (FGF-2)), matrix remodeling effectors (such as MMP-25) and inflammatory signals (such as chemokine (C-C motif) ligand 2 (CCL2)). CCL2 can act on tissue resident monocytes to induce differentiation and production of inflammatory cytokines such as IL-6 and tumor necrosis factor-α (TNF-α). TNF-α can then act on tenocytes to inhibit collagen deposition and release matrix remodeling enzymes, promoting inflammatory debridement of the injured area. TNF-α and IL-6 can induce the production of IL-10 [2], which can initiate a negative feedback loop, suppressing the damaged induced inflammatory response

response in tendon is likely integrated into a mechanosensing apparatus capable of detecting injurious mechanical forces. This field of study is underrepresented in the literature [77], but studies have elucidated some of the major players in the system (Fig. 7.5).

Tenocytes in isolated tendons exposed to forces capable of producing overt microstructural damage expressed IL-1β and MMP-13. Results of IL-1β siRNA transfection experiments demonstrated that MMP-13 expression was at least partially dependent on expression with IL-1β, suggesting an autocrine/paracrine role for IL-1β in tendon [95]. In a separate study, in an *ex-vivo* bovine model, stretch induced a coordinated pro-inflammatory response across multiple classes of distinct, yet unidentified, cells in tendon. Upon a stretch challenge, the matrix degrading enzymes MMP-1 and C1,2C were found expressed by putative tenocytes near microtears. At the same time, immunohistochemical analysis revealed two separate unidentified cell populations located near damaged tissue expressed IL-6 or COX-2 [93]. These mediators have the potential to not only initiate an inflammatory response, but also have the capacity to initiate tendon repair. IL-6 was required for tendon repair in a knockout mouse model [67], and PGE_2, a product of the COX-2 arachidonic acid pathway, increased tendon strength in a rat treatment model [43].

Part of the link between IL-1β and the observed downstream inflammatory effects may be TNF-α. TNF-α has an array of effects on tenocytes in culture including blocking the production of collagen I, stimulating the production of MMP-1, and (in conjunction with IL-6) causing the production of immunoregulatory IL-10 [53]. Increased matrix metalloprotease expression combined with reduced collagen expression may promote inflammatory infiltration of an injured tissue. Coordinated activity of IL-10 and IL-6 in an inflammatory environment can potentially suppress runaway inflammatory responses due to matrix damage caused by infiltrating inflammatory cells. Continued suppression, possibly combined with an influx of T-cells due to IL-15 production (see Table 7.1), can potentially lead to chronic tendon injury observations, such as expression of insulin like growth factor and suppressors of cytokine signaling [3], and eventually lead to repair responses [52].

Taken together, these findings describe an initiation of inflammatory debridement of injured tissue, complete with regulatory steps that can suppress damage-induced inflammatory insult in response to TNF-α. However, although some of the downstream inflammatory actors have been identified and associated with IL-1β, the link between IL-1β production and TNF-α production in tendon is not well understood. As part of an effort to dissect this link in tendon, our lab exposed primary human tenocytes in culture to IL-1β and performed microarray analysis. GO analysis [38]

Table 7.1 Primary human tenocytes isolated from three human flexor carpi radialis tendons were exposed to 100 pM interleukin-1β for 24 h *in vitro*

Gene	Fold increase	Function
		Inflammation
CCL2	6.5	Chemotactic factor for monocytes and basophils
IL1β	6.0	Activated by caspase 1. Wide array of activities including proliferation and differentiation.
CXCL3	5.5	Chemokine for neutrophils
CCL7	5.4	Chemokine for macrophages.
CCL8	5.3	Chemokine for multiple inflammatory cells
IFNA8	4.5	Interferon in the TGFβ pathway.
TSLP	4.1	Induces monocyte-mediated recruitment of T-cells
BCL2A1	4.0	Inhibits release of cytochrome c. Apoptosis-protective
BDKRB1	3.8	Receptor. Responds to inflammatory responses to tissue damage
VNN2	3.1	Promotes neutrophils migration
CXCL10	2.9	Chemokine for monocytes. Promotes cell adhesion.
TNFRSF11β	2.9	Decoy receptor. Blocks bone resorption
IL15	2.5	T-cell regulator. Promotes survival via BCL2
		Proliferation & Repair
EREG	5.1	Epiregulin. Ligand for epidermal growth factor receptor.
TNFAIP6	5.1	Matric binding factor that promotes matrix stability
LAMA4	4.2	Laminin 4, matrix protein
FGF2	3.6	Fibroblast mitogenic factor. Promotes migration and proliferation.
PLK2	3.0	Important in proliferation
CDK6	2.8	Allows G1 progression
PLA2G3	2.8	Secreted phospholipase. Generates arachidonic acid.
LOXL4	2.5	Metabolic enzyme essential in matrix crosslinking
LAMA3	2.1	Laminin 3, matrix protein
PLA2G4	2.0	Cytosolic phospholipase. Generates arachidonic acid.
		Remodeling
HABP2	3.8	Serine protease that cleaves fibrinogen. Binds to matrix and is involved in adhesion.
NINJ1	3.5	Adhesion molecule. Role in wound healing.
ADAM11	3.3	Promotes cell-cell-matrix interactions. Important in tissue repair and development
MMP25	2.8	Metalloprotease. Promotes inflammatory cell invasion of tissue
SOST	2.7	BMP antagonist
CD47	2.3	Cell adhesion signaling molecule
COL17A1	2.2	Hemidesmosome component

RNA was collected and analyzed by Agilent RNA array. Key upregulated genes associated with inflammation, remodeling, and proliferation and repair are shown.

indicated strong enrichment for cytokine-mediated signaling pathways (GO:0019221, $p = 2 \times 10^{-6}$), positive regulation of cytokine production (GO:0001819, $p = 6 \times 10^{-5}$), and inflammatory responses (GO:0006954, $p = 6 \times 10^{-6}$).

An array of inflammatory signals were up-regulated in human tenocytes isolated the flexor carpi radialis (Table 7.1), showing that autocrine/paracrine IL-1β signaling can lead to the production of a powerful inflammatory cocktail. Concurrently, the increased production of genes associated with remodeling can allow for the infiltration of inflammatory cells and promote enzymatic degradation of damaged matrix following injury. TGF-β3 expression was reduced more than sevenfold after IL-1β stimulation, which would reduce the production and deposition of collagen I and III [58]. Expression of secreted (PLA2G3) and cytosolic (PLA2G4) phospholipases supports the role of COX-2 and

PGE_2 in tendon repair. Expression of matrix laminin and growth factors (such as EREG and FGF-2) rounds out the beginnings of a set of controlled signaling sequences leading from inflammation to resolution to repair.

Conclusion

Further research revealing how tenocytes, *in vitro* and *in vivo*, respond to strain and ligands in health and disease will be revealed as the field focuses on altered pathways discerned from array and metabolomics data. For tendon biology, fundamental signaling pathways and well known pathways in strain responses and inflammation have been elucidated. Future work must reveal pathways that can be manipulated to prevent matrix degradation and even support functional matrix replacement with muscle, bone and nerve sparing strategies. Further studies are also needed to better elucidate the role of tendon-derived stem cells, a potential source for endogenous repair [65], and the role these cells play in tendon development, tendinopathy, rehabilitation as well as mechanotransduction [20, 80, 106].

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