3 Tendon Extracellular Matrix Assembly, Maintenance and Dysregulation Throughout Life

Seyed Mohammad Siadat, Danae E. Zamboulis, Chavaunne T. Thorpe, Jeffrey W. Ruberti, and Brianne K. Connizzo

Abstract

In his Lissner Award medal lecture in 2000, Stephen Cowin asked the question: "How is a tissue built?" It is not a new question, but it remains as relevant today as it did when it was asked 20 years ago. In fact, research on the organization and development of tissue structure has been a primary focus of tendon and ligament research for over two centuries. The tendon extracellular matrix (ECM) is critical to overall tissue function; it gives the tissue its unique mechanical properties, exhibiting complex non-linear responses, viscoelasticity and flow mechanisms, excellent energy storage and fatigue resistance. This matrix also creates a unique microenvironment for resident cells, allowing cells to maintain their phenotype and translate mechanical and chemical signals into biological responses. Importantly, this architecture is constantly remodeled by local cell populations in response to changing biochemical (systemic and local disease or injury) and mechanical (exercise, disuse, and overuse) stimuli. Here, we review the current understanding of matrix remodeling throughout life, focusing on formation and assembly during the postnatal period, maintenance and homeostasis during adulthood, and changes to homeostasis in natural aging. We also discuss advances in model systems and novel tools for studying collagen and non-collagenous matrix remodeling throughout life, and finally conclude by identifying key questions that have yet to be answered.

Keywords

Tendon · Collagen remodeling · Non-collageneous matrix · Homeostasis · Aging

S. M. Siadat · J. W. Ruberti
Department of Bioengineering, Northeastern University, Boston, MA, USA
e-mail: siadat.s@northeastern.edu; j.ruberti@northeastern.edu

D. E. Zamboulis
Institute of Life Course and Medical Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, UK
e-mail: D.E.Zamboulis@liverpool.ac.uk

C. T. Thorpe
Comparative Biomedical Sciences, The Royal Veterinary College, University of London, London, UK
e-mail: cthorpe@rvc.ac.uk

B. K. Connizzo (✉)
Department of Biomedical Engineering, Boston University, Boston, MA, USA
e-mail: connizzo@bu.edu

J. Halper (ed.), *Progress in Heritable Soft Connective Tissue Diseases*, Advances in Experimental Medicine and Biology 1348, https://doi.org/10.1007/978-3-030-80614-9_3

Abbreviations

AGEs	Advanced glycation end-products
Aha	Azidohomoalanine
BATs	Bioartificial tendons
CHP	Collagen hybridizing peptide
CMP	Collagen mimetic peptide
COMP	Cartilage oligomeric matrix protein
CSA	Cross sectional area
DAMPs	Damage-associated molecular patterns
DIBO	Dibenzooctyne
DIC	Differential interference contrast
DTAF	Dichlorotriazinyl aminofluorescein
ECM	Extracellular matrix
EDS	Ehlers-Danlos Syndrome
EM	Electron microscopy
FACIT	Fibril-associated collagens with interrupted triple helices
FIC	Flow-induced crystallization
FN	Fibronectin
FRET	Förster resonance energy transfer
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GPC	Golgi to plasma membrane carrier
IL	Interleukin
LEs	Ligament equivalents
Met	Methionine
MMP	Matrix metalloproteinase
N_3-Pro	Azido-proline
PGE_2	Prostaglandin E_2
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SHG	Second harmonic generation
SLRP	Small leucine rich proteoglycan
TECs	Tissue engineered constructs
TEM	Transmission electron microscopy
TSCs	Tendon stem cells
TSP	Thrombospondin

3.1 Introduction

In his Lissner Award medal lecture in 2000, Stephen Cowin asked the question: "How is a tissue built?" It is not a new question, but it remains as relevant today as it did when it was asked 20 years ago (Cowin 2000). In fact, research on the organization and development of tissue structure has been a primary focus of tendon and ligament research for over two centuries. The tendon extracellular matrix (ECM) is critical to overall tissue function; it gives the tissue its unique mechanical function, exhibiting complex nonlinear responses, viscoelasticity and flow mechanisms, excellent energy storage and fatigue resistance (Butler et al. 1997; Connizzo et al. 2013a; Franchi et al. 2007; Thorpe and Screen 2016; Thompson et al. 2017). This matrix also creates a unique microenvironment for resident cells, allowing cells to maintain their phenotype and translate mechanical and chemical signals into biological responses (Thompson et al. 2017; Wall et al. 2018; Wang et al. 2013a; Dyment et al. 2020). Importantly, this architecture is constantly remodeled by local cell populations in response to functional changes such as exercise, as well as in response to tissue damage or injury. Here, we review our current understanding of matrix remodeling throughout life, focusing on formation and assembly during the postnatal period, maintenance and homeostasis during adulthood, and changes to homeostasis in natural aging.

3.1.1 Tendon Composition, Structure, and Function

The dry weight of the tendon ECM can be dissected into two main components: the collagenous structural hierarchy, and the non-collagenous matrix (Fig. 3.1). Both components are essential to tendon function and biology, although the collagenous structure has been studied far more extensively. Type I collagen is the primary protein in tendon, accounting for 65–80% of the dry mass of the tendon (Brinckmann and Bachinger 2005; Kannus 2000). The asymmetric triple-helix collagen molecules coil to form the triple helix of a collagen molecule (Mienaltowski and Birk 2014). Collagen molecules then link in a quarter staggered orientation to form fibrils. Collagen fibrils, now considered to be the basic unit of tendon, are bundled together within a collagen fiber. Collagen fibers are then bundled together and bound via a fine sheath of tissue; this structure is now called a fascicle. Fascicles then bundle to

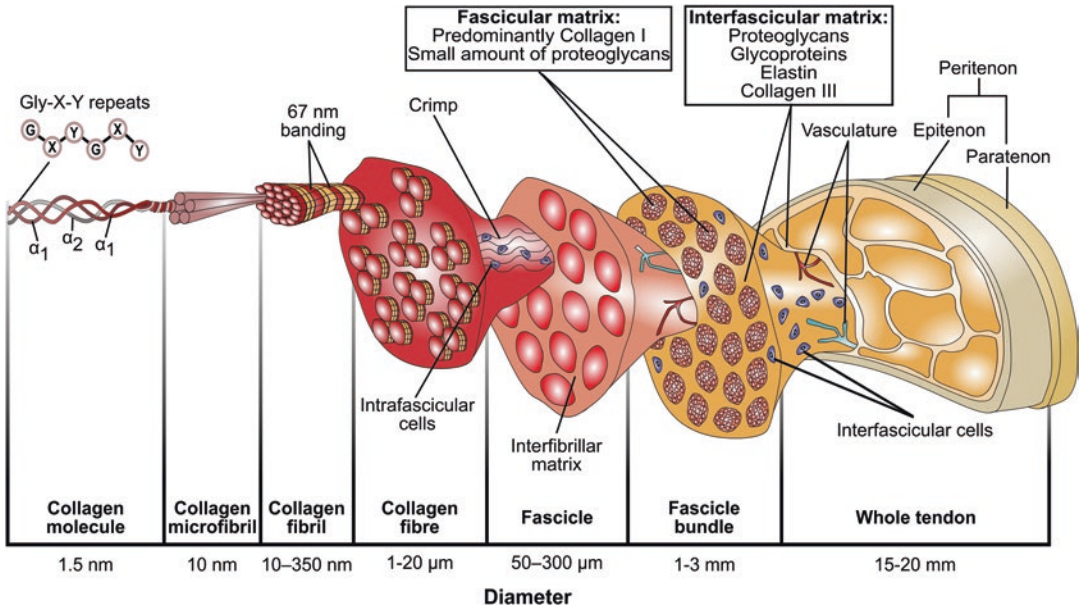

Fig. 3.1 Hierarchical organization of the equine superficial digital flexor tendon with specific detail related to the interfascicular and interfibrillar matrix composition. (Reproduced from O'Brien et al. 2020)

form whole tendon, which is surrounded by the epitenon sheath. The non-collagenous matrix in tendon is found interspersed between collagen fibrils, fibers, and fascicles in the interfibrillar, interfiber, and interfascicular region of the tendon, respectively, and is mainly composed of proteoglycans, glycoproteins, and minor collagens (Kannus et al. 1998; Taye et al. 2020; Thorpe et al. 2016a).

The structural organization of the tendon ECM is a major contributor to overall tissue function. During mechanical loading, collagen fascicles, fibers, and fibrils exhibit a number of dynamic responses that allow for reduction of stress concentrations and prevent structural damage (Connizzo et al. 2013a; Franchi et al. 2007). This includes uncrimping (Lavagnino et al. 2017; Patterson-Kane et al. 1997; Miller et al. 2012a), or the reduction in the wavy formation of the collagen fibers, and fiber/fibril re-alignment (Miller et al. 2012b; Connizzo et al. 2013b; Lake et al. 2010), when these structures re-orient towards the axis of loading and consolidate to a single fiber direction. In addition, collagen fascicles, fibers, and fibrils have all demonstrated the capacity to slide against one another, although this ability is more often attributed to the properties of the non-collagenous compartment rather than the collagen structure itself (Connizzo et al. 2014a; Rigozzi et al. 2013; Thorpe et al. 2015a; Szczesny and Elliott 2014). In addition, proteoglycans and their glycosaminoglycan (GAG) chains present in the non-collagenous compartment attract and trap water molecules allowing for complex fluid flow and viscoporoelasticity (Butler et al. 1997; Rigozzi et al. 2013; Legerlotz et al. 2013a; Connizzo and Grodzinsky 2017; Buckley et al. 2013). It is crucial to note however that both the structure and function of tendons and ligaments varies significantly based on tissue site, and more specifically based on the functional demands of the tissue.

3.1.2 Function-Based Variations in Tendon Composition and Structure

All tendons within the appendicular skeleton transfer muscle-generated force to the bony skeleton, positioning the limbs during locomotion. In addition to a positional function, specific tendons also store and release energy as they stretch and recoil with each stride, reducing the energetic

cost of locomotion (McNeill 2002). The major energy storing tendons in the human are the Achilles and hamstring tendons, whereas in large quadrupeds, such as the horse, the digital flexor tendons are the predominant energy storing tissues (Shepherd et al. 2014; Lichtwark and Wilson 2005; Biewener 1998). Energy storing tendons require specialised mechanical properties for their function, including greater compliance and enhanced fatigue resistance, properties that are conferred by compositional and structural specialisations at different levels of the tendon hierarchy (Thorpe and Screen 2016; Thorpe et al. 2013a). Here, we specify research performed in energy storing or positional tendons for clarity wherever relevant.

Tendon structure and composition are also dramatically different at the junction with muscle and bone compared to the midsubstance. The enthesis, or insertion site, has unique compositional and structural properties that allow it to minimize stress concentrations at the junction of dissimilar materials (Deymier-Black et al. 2015; Thomopoulos et al. 2003; Saadat et al. 2016). Tissue function at these sites is also altered, demonstrating more complex multi-scale mechanical responses (Connizzo et al. 2016a). In addition, some tendons exhibit unique anatomical positions that alter function. Tendons that wrap around bony structures exhibit cartilaginous-like tissue regions with higher levels of the large proteoglycan aggrecan and enhanced mechanical function in compression (Connizzo and Grodzinsky 2018a; Wren et al. 2000; Koob and Vogel 1987; Fang et al. 2014). For the purposes of this discussion, we focus on general changes across multiple species in the collagen structure and non-collagenous matrix at the midsubstance of the tendon and not in specialized regions.

3.1.3 Tendon Cell Populations

Remodeling of the extracellular matrix is cell-mediated, and therefore an understanding of cell populations within tendon is necessary for discussion of this highly complex process. Early in development, tendon is highly cellular, with proliferative cells appearing homogenous with more rounded cell nuclei. Following deposition of the extracellular matrix, tendon becomes hypocellular with limited mitotic activity and a heterogeneous cell population with cells with long and spindle shaped nuclei in the fascicles and the more rounded, densely packed cells in the interfascicular matrix (Oryan and Shoushtari 2008; Russo et al. 2015; Grinstein et al. 2019; Zamboulis et al. 2020). Until recently the main cell types that had been described in tendon were tenocytes and tendon progenitor/stem cells (TSCs) as well as tissue-resident immune cells, vascular cells, neuronal cells, and chondrocyte-like cells at the tendon insertion (Kannus 2000; Ackermann et al. 2016; Thomopoulos et al. 2010; Bi et al. 2007; Lee et al. 2018; Mienaltowski et al. 2018). With the advent of single-cell sequencing, the investigation of cell heterogeneity within tissues has been made possible and its recent use in tendon research has unveiled several tendon cell subtypes (Paolillo et al. 2019; Harvey et al. 2019; Kendal et al. 2020; De Micheli et al. 2020; Yin et al. 2016), but the role of the identified clusters in the development, maintenance, and aging of tendon still remains to be elucidated.

3.2 Postnatal Development

3.2.1 Collagen Fibril Formation

The highly dynamic nature of fibrillogenesis and growth of fibrils in the complex extracellular environment has made it challenging to precisely separate the events that cause conversion of soluble collagen to an insoluble fibril. *In vitro* polymerization of tissue-extracted collagen molecules in solution has shed light on fibrillogenesis kinetics and thermodynamics. Collagen molecules polymerize spontaneously at physiological pH, temperature, and ionic strength (Gross and Kirk 1958; Wood 1964; Williams et al. 1978; Vanamee and Porter 1951) demonstrating the same detailed fine structure of native fibrils (Vanamee and Porter 1951; Bahr 1950; Noda and Wyckoff 1951; Schmitt et al. 1942). Slight deviations from

physiological conditions lead to formation of abnormal fibrils (Gross 1956). Thermodynamically, type I collagen fibrillogenesis *in vitro* is an entropy-driven and endothermic self-assembly process (Kadler et al. 1987) which is driven by the loss of solvent molecules from the collagen surface. *In vitro* self-assembly, however, cannot explain the formation of highly organized native collagenous tissues such as tendon with a multi-hierarchical structure comprising molecules, fibrils, fibers, and fascicles all parallel to the long axis of the tendon (Franchi et al. 2007). Formation of unorganized networks of fibrils varying in diameter and direction *in vitro* (Wood and Keech 1960; Bard and Chapman 1973), points to the critical role of cellular environment *in vivo*. It is clear that collagen production and fibrillogenesis is under the direct control of fibroblasts (Wolbach and Howe 1926; Maximow 1928; Stearns 1940a, b; Wassermann 1954; Porter and Pappas 1959; Chapman 1961; Peach et al. 1961; Ross and Benditt 1961; Goldberg and Green 1964). What is not exactly clear, is the site and mechanism of initial fibril formation which has been the subject of studies for almost two centuries (Schwann 1839, 1847). The literature contains contradictory explanations regarding whether the collagen fibrils of the connective tissues arise within the cytoplasm (Ferguson 1912; Bradbury and Meek 1958; Godman and Porter 1960), on the surface (Porter and Pappas 1959; Mall 1902), or in the intercellular spaces (Stearns 1940a, b; Ross and Benditt 1961; Mallory 1903; Hertzler 1910; Baitsell 1915, 1916, 1921, 1925; Isaacs 1916, 1919; Gross et al. 1955; Ross and Benditt 1962) of collagen-secreting cells.

After the advent of electron microscopy, several studies demonstrated vesicular components containing small fibrils just below the cell surface (Bradbury and Meek 1958; Godman and Porter 1960; Sheldon and Kimball 1962; Voelz 1964; Welsh 1966; Trelstad 1971). High voltage electron microscopy revealed collagen fibrils within small surface recesses in chick embryo cornea (Birk and Trelstad 1984), tendon (Birk and Trelstad 1986; Yang and Birk 1986), and dermis (Ploetz et al. 1991) fibroblasts. It was suggested that cells directly produce fibrils within these deep and narrow recesses and place them into the ECM (Fig. 3.2a). However, it was previously shown that fibrils can be produced by any action that causes shrinkage of the intercellular substance (Isaacs 1919), increasing the possibility of formation of artificial fibrils due to fixation or dehydration in prepared samples for electron microscopy. Canty et al. (2004) using serial section and 3-D reconstructions of chick embryonic tendon fibroblasts revealed fibrils within closed intracellular Golgi to plasma membrane carriers (GPCs). Further, using pulse-chase experiments, procollagen fragments were detected within the GPCs (Canty et al. 2004). It was proposed that the GPCs were on their way to plasma membrane protrusions, which were named fibril depositors or fibripositors. It has been widely accepted now that fibripositors are the site of fibril assembly *in vivo* (Holmes et al. 2018); fibril segments are formed intracellularly and then discharged into extracellular space by the non-muscle myosin II mechanism (Fig. 3.2b) (Kalson et al. 2013; Canty et al. 2004).

However, fibripositors are absent during postnatal development (Humphries et al. 2008) and therefore cannot explain the persistent production of *de novo* fibrils in postnatal tendon and throughout life (Chang et al. 2020) when cells lose their ability to directly access damaged or developing fibrils in the dense and mature ECM (Isaacs 1919; Kalson et al. 2015). The fibripositor theory is also unclear regarding intracellular processing of procollagen. It has been shown that removal of the carboxyl propeptides lowers the solubility of procollagen (Kadler and Watson 1995) and is an essential step for the assembly of collagen fibrils (Prockop et al. 1979a, b). While procollagen processing has been reported within intracellular compartments of postnatal murine (Humphries et al. 2008) and chick embryonic (Canty et al. 2004) tendon fibroblasts, the enzymes for procollagen cleavage have been detected primarily within the extracellular culture medium (Hojima et al. 1985; Kessler and Goldberg 1978; Duksin et al. 1978; Leung et al. 1979; Jimenez et al. 1971) and not extracts of the cells (Goldberg et al. 1975). The required ionic

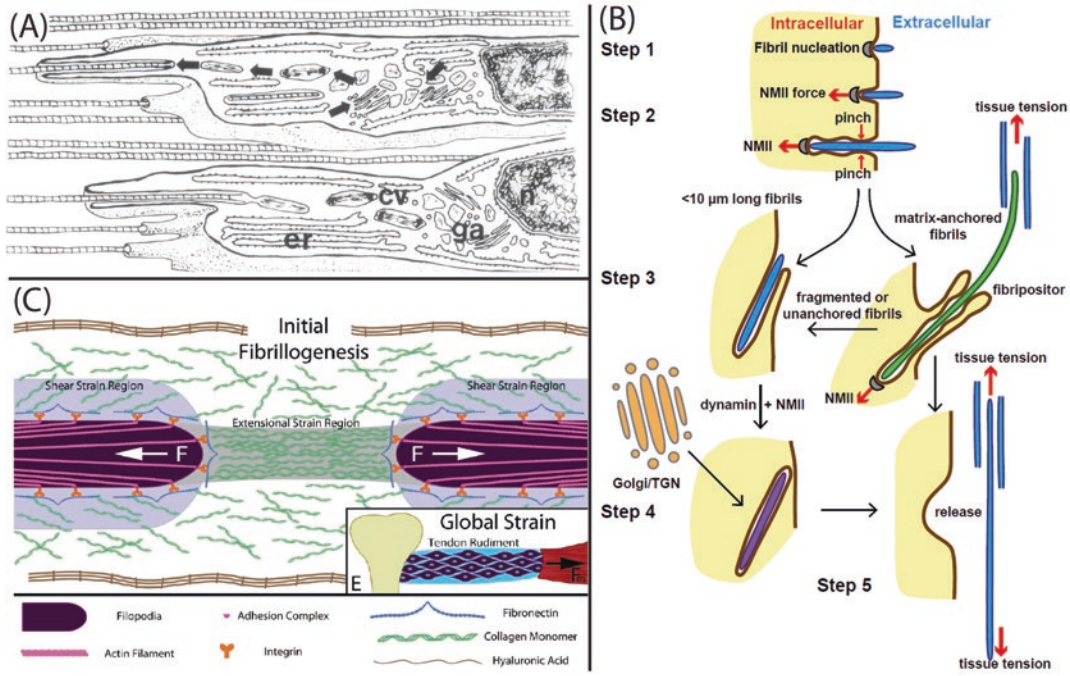

Fig. 3.2 Possible mechanisms of fibril formation. (**A**) Collagen fibrillogenesis model proposed by Trelstad and Hayashi (1979). Collagen is synthesized in the endoplasmic reticulum (er), packaged in the Golgi apparatus (ga), and transferred in condensation vacuoles (cv) to deep cytoplasmic recesses (site of fibril assembly). (**B**) The processes of collagen fibril nucleation and movement in the fibripositor model proposed by Kalson et al. (2013). The initial collagen fibril nucleation occurs at the plasma membrane by accretion of collagen molecules or collagen aggregates. NMII powers the transport of newly formed fibrils in fibripositors. (**C**) Flow-induced crystallization model by Paten et al. (2016) elucidating the early stage of tendon morphogenesis *in vivo*: (1) cell recruitment, (2) cell migration and organization, (3) ECM molecular synthesis e.g., collagen monomers, fibronectin, elastin, proteoglycans and hyaluronic acid, (4) initial fibrillogenesis by filopodia on the fibroblasts via exerting a contractile force on collagen-binding complexes, and (5) tissue strains cause formation of additional fibrils precisely where they are required for tissue connectivity

calcium concentration for enzyme activity (Hojima et al. 1985) is also orders of magnitude larger than intracellular calcium concentration (Bronner 2001). Furthermore, the procollagen proteinases are neutral metalloproteinases (Kessler and Goldberg 1978; Duksin et al. 1978; Leung et al. 1979; Goldberg et al. 1975; Njieha et al. 1982; Bornstein et al. 1972) and have negligible activity at pH 6 or below (Hojima et al. 1985, 1994). The acidic pH of Golgi network transport carriers and secretory vacuoles (Demaurex et al. 1998) is incompatible with the neutral pH condition required for procollagen processing and fibrillogenesis of collagen molecules. N'Diaye et al. recently showed that the extracellular space is the main action site of bone morphogenetic protein 1, which is required for type I procollagen C-terminal processing in postnatal lung fibroblasts (N'Diaye et al. 2020). It is possible that the detected intracellular collagen fragments in other studies (Canty et al. 2004; Humphries et al. 2008) are processed extracellularly and then rapidly endocytosed.

Several studies suggest that intracytoplasmic fibrils are evidence for the ability of fibroblasts to phagocytose extracellular collagen fibrils in rapidly remodeling (Ten Cate 1972; Ten Cate and Deporter 1974, 1975; Ten Cate and Freeman 1974; Listgarten 1973) or developing (Dyer and Peppler 1977) tissues. Intracellular mature fibrils

have been reported with loss of banding (Ten Cate 1972), coiled in membrane-bound structures (Ten Cate 1972), and with poorly-visualized structures (Listgarten 1973). Some fibrils were observed situated partly within the fibroblast and partly outside of it while demonstrating the presence of enzyme activity (Deporter and Ten Cate 1973). All of this suggests that the observed intracellular fibrils were once extracellular and on their way to be degraded intracellularly. It has been shown that intracellular cross-banded collagen fibrils appear even when collagen synthesis is blocked (Everts et al. 1985; Everts and Beertsen 1987; Beertsen et al. 1984) and that cytoplasmic actin filament systems are involved in the phagocytosis of collagen (Everts et al. 1985, 1989). Furthermore, quantitative radio-autography after injection of ^{3}H-proline revealed that collagen precursors (procollagen) were released outside of the cell fibroblasts (Marchi and Leblond 1983, 1984). The observed intracytoplasmic collagen fibrils did not contain the new labeled proline, but were instead associated with lysosomes and digestive vacuoles, had lost their banding and were at various stages of degeneration.

Several studies suggest that fibril formation could operate independently of the cell surface or at some nominal distance from it, guided by long-range spatial cues provided by cell traction (Stopak et al. 1985) or mechanical forces (Gross et al. 1955; Paten et al. 2016; Lewis 1917). Wolbach followed histologic sequences in the development of connective tissue of guinea pigs under a scorbutic condition (Wolbach and Howe 1926; Wolbach 1933). It was suggested that rapid appearance and large volume of intercellular collagen fibrils is due to presence of a liquid precursor of collagen in the extracellular space, and that the collagen fibril formation is influenced by forces acting on this homogeneous collagen. Another study followed the progress of a healing wound in the connective tissue of a living rabbit's ear, demonstrating that intercellular connective tissue fibrils formed extracellularly as a result of fibroblastic activity (Stearns 1940a, b). The fibroblasts participated directly in the process by the projection of cytoplasmic material from their surface. Since this cytoplasmic material disappeared as the fibrils formed, it was suggested that the secreted material was utilized in the production of fibrils guided by applied tension and orientation of fibroblast cells. Emerging evidence suggests the presence of a newly synthesized precursor – tropocollagen – that is free in the ECM (Gross et al. 1955) and diffuses away from the secretory cells (Revel and Hay 1963), and that individual collagen fibrils can form from precursor molecules/microfibrils produced by more than one cell (Lu et al. 2018).

Paten et al. demonstrated *in vitro* how tension can directly drive initial fibrillogenesis (Paten et al. 2016). It was shown that organized fibrils can be formed by slowly drawing a microneedle from the slightly concentrated surface of a collagen solution droplet. They then proposed a model for early connective tissue development in which extensional strain triggers fibril formation extracellularly directly in the path of force. Paten et al. further expanded the concept to address the establishment of continuity in collagenous tissue, suggesting that the amplification of the extensional strain rate between the ends of early fibrils can rapidly fuse them by flow-induced crystallization (FIC) (Fig. 3.2c). They further estimated that the required collagen concentration and contraction rates necessary for FIC is achievable by the local cell population. While it has not yet been demonstrated experimentally, the FIC model has the potential to explain (1) the abundance of short fibril segments during initial tendon morphogenesis and their end-to-end growth (Birk et al. 1995, 1997), (2) the synchronized alignment of collagen fibrils far from the main cell body (Young et al. 2014), and (3) the role of hyaluronic acid (Goldberg and Green 1964; Green and Hemerman 1964), fibronectin (Sottile and Hocking 2002; McDonald et al. 1982; Paten et al. 2019), actin filaments (Johnson and Galis 2003), and integrins (Li et al. 2003) which have been all shown previously to be necessary for collagen fibrillogenesis. While the precise manner in which collagen molecules are manipulated to drive the formation, growth, and remodeling of collagen fibrils has not been agreed upon, it is likely guided by a common physical and regulated by multiple factors to establish long-range

connectivity and growth of collagenous structures into the path of force, where it is needed.

3.2.2 Post-formation Assembly

Embryonic growth occurs by an increase in both fibril number and diameter (Parry and Craig 1977, 1978; Scott et al. 1981; Scott and Hughes 1986). In the postnatal period, tendon growth continues by increases in fibril diameter and length (Parry and Craig 1977, 1978; Parry et al. 1978a; Eikenberry et al. 1982; Michna 1984) in a multi-stage growth/stabilization process (Nurminskaya and Birk 1998). The manner in which molecules or fibril segments add to the growing fibril *in vivo* is not completely understood. Fibril growth involves both an intrinsic self-assembly process (diffusion-controlled) and extrinsic regulation (interface-controlled) by other fibril-associated molecules, and the local environment of collagen fibrils (Hoffmann et al. 2019). The data from growing native fibrils have provided evidence for models of fibril fusion (Graham et al. 2000; Kadler et al. 2000), molecular accretion (Kalson et al. 2015; Holmes et al. 2010), and possibly a combination of both (Birk et al. 1997; Ezura et al. 2000).

Interfibrillar fusion can potentially involve tip-to-tip, tip-to-shaft, and shaft-to-shaft fusion (Birk et al. 1995). However, bipolar fibrils with two C-ends or fibrils with multiple switch regions have not been found, either *in vivo* or *in vitro* (Fig. 3.3) (Kadler et al. 1996). End-to-end fusion of unipolar and bipolar fibrils will decrease the unipolar fibril population. Therefore, an enriched bipolar fibril population, unable to fuse further, could determine the limit of fibril growth in length. Fibril fusion can also be regulated by fibril-associated proteoglycans or some other macromolecule through maintaining interfibrillar spacing and inhibition of lateral segment fusion (Scott et al. 1981). It has been shown that mature rat tail tendon comprises several fibrils in the process of fusion or separation with some intrafibrillar proteoglycans inside large collagen fibrils (Scott 1990). Furthermore, fibrils' tips in embryonic chick metatarsal leg tendons have less surface bound proteoglycans compared to the fibril shaft allowing for tip-to-tip fusion and longitudinal fibril growth (Graham et al. 2000).

Direct evidence for molecular accretion *in vivo* is scarce due to the difficulty of visualizing and tracking of single collagen molecules (see Sect. 3.5.3). It has been shown that slow stretching of a cell culture tendon-like construct increases fibril diameter and volume fraction (Kalson et al. 2011). However, interfibrillar fusion alone could not explain the increase in fibril volume fraction. *In vitro* studies have also shown direct evidence for growing fibrils from acid-soluble collagen (Holmes and Chapman 1979). Fractured ends of isolated fibrils from avian embryonic tendon can further grow in the opposite axial direction by molecular accretion (Holmes et al. 2010). Kalson et al. (2015) presented a growth model based on 3D-electron microscopy of mouse tail tendon (Kalson et al. 2015). During the embryonic growth stage, fibril number, diameter, and length increase by fibril nucleation and axial growth. During postnatal growth, fibril number remains constant but fibril diameter and length continue to grow likely by molecular accretion. Birk et al. (1997) proposed a model in which thin fibril intermediates are formed by molecular accretion in chicken embryo metatarsal tendon (Birk et al. 1997). Then, longer and larger diameter fibrils are produced by lateral associations of preformed segments. The longer fibrils would have multiple polarity changes which would determine the regions able to associate. Growth would follow by molecular rearrangement to reconstitute cylindrical fibrils. Enzymatic intervention is also considered in this model to degrade poorly cross-linked fibrils in regions of polarity reversal and generate short polar units that could participate in further growth. Ezura et al. (2000) also suggested a fibril growth model in the developing mouse flexor tendons where fibril intermediates form by molecular accretion and are stabilized through their interactions with small leucine-rich repeat proteoglycans (Ezura et al. 2000). The change in composition of the matrix proteoglycans leads to a multi-step fusion/growth process. More tissue specific models are needed to fully explain the

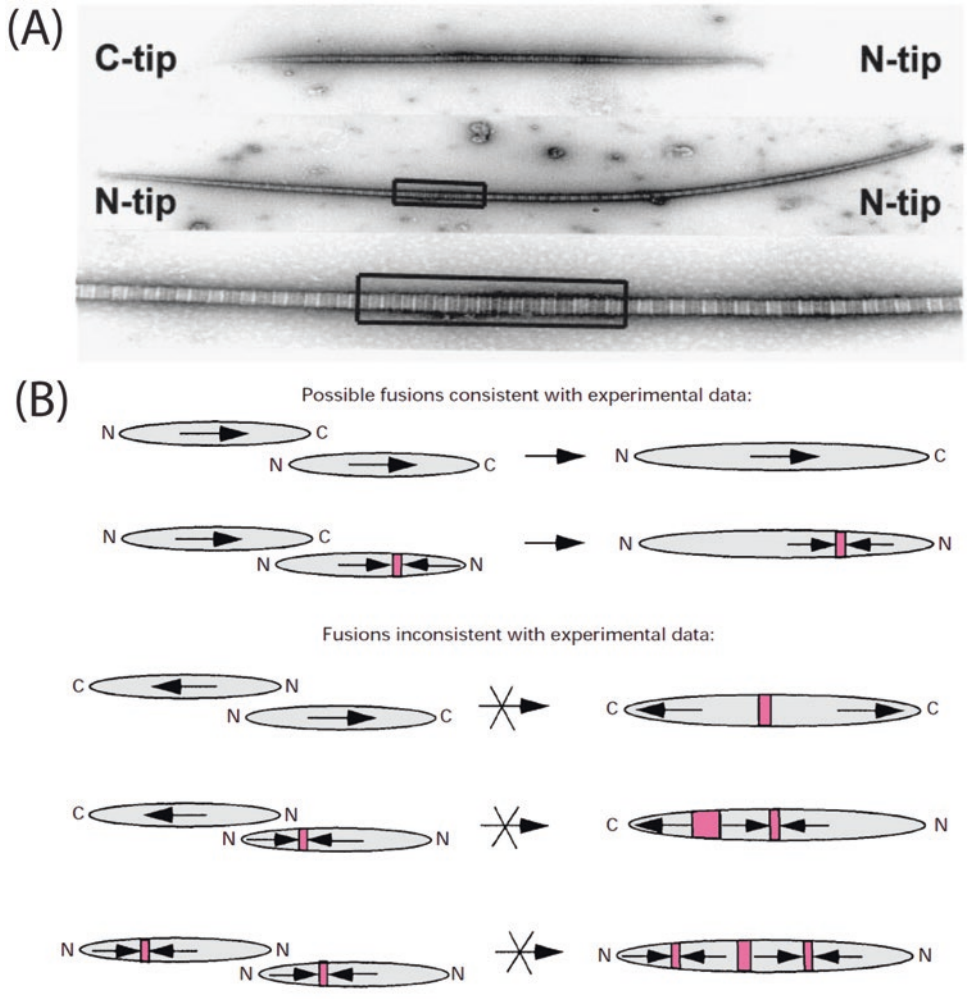

Fig. 3.3 Collagen fibril polarity and fusion. (**a**) Unipolar and bipolar collagen fibrils from embryonic chick tendon. Reproduced with permission from Kadler et al. (2000). The molecular switch region of a bipolar fibril is shown in magnification. (**b**) Possible models of fibril end-to-end fusion based on fibril's polarity. Arrows indicate molecular polarity within a fibril and pink boxes indicate regions of polarity reversal. Reproduced from Kadler et al. (1996)

combination of fibril associated molecules in every stage of fibril growth and stabilization which establishes the biological and mechanical functionality of tendons (Robinson et al. 2005).

Fibril growth mechanisms might be different in tissues with different mechanical and biological functions. For example, fibrils from sea cucumber dermis (Trotter et al. 1998) and sea urchin spine ligament (Trotter et al. 2000) display symmetrical mass distributions with a single transition zone in the center, making fibril fusion an unlikely growth mechanism (Trotter et al. 1998). Most likely, fibril growth throughout life in tendon is maintained by molecular accretion as well as linear and lateral association of fibril segments. In the early stages of development, tissue architecture is defined by fibril growth in number and length possibly through flow-induced crystallization (Paten et al. 2016) and/or spontaneous end-to-end fusion of small fibril segments

(Graham et al. 2000). Later in development and upon removal of lateral growth inhibitors, fibrils rapidly grow by lateral fusion (Scott et al. 1981) followed by molecular accretion to maintain a uniform (Parry and Craig 1984), energetically-stable shape. Cross-linked, adult fibrils may grow and remodel further by molecular accretion upon mechanical loading or injury of tendon.

3.2.3 Regulators of Matrix Growth and Development

Regardless of the mechanism, fibril growth in tendon and ligaments is highly regulated (Parry et al. 1978b). Fibrils *in vivo* are cylindrical with uniform diameter (Parry and Craig 1984), but reconstituted fibrils *in vitro* have a broad diameter distribution (Bard and Chapman 1973). Presence of an upper limit for fibril diameter may be due to the difficulty of the addition of new molecules or fibril segments and points to the participation of several regulatory processes, detailed below.

3.2.3.1 Water Structures

Collagen structure and stability is driven by molecular interaction with water molecules (Finch and Ledward 1972; Luescher et al. 1974; Kopp et al. 1990; Bigi et al. 1987; Miles and Ghelashvili 1999; Na 1989; Tiktopulo and Kajava 1998; Burjanadze 1982). Initial fibril formation is an endothermic, but entropy driven process (Kadler et al. 1987; Cassel 1966) arising from release of water molecules (Streeter and de Leeuw 2011; Kauzmann 1959). Post formation assembly can also be regulated by stabilization of water molecules (Cooper 1970), where breakers of water structure promote fibril formation, and makers of water structure are inhibitory (Hayashi and Nagai 1972). Mature fibrils *in vivo* are cross-linked by covalent bonds between neighboring molecules. However, the young and growing fibrils are stabilized by non-covalent hydrogen bonds (Bailey et al. 1998) and have the potential to bind more water molecules (Kopp et al. 1990). In fact, proteoglycans (Birk et al. 1996) or hyaluronate (Scott et al. 1981; Scott 1984) can stabilize the water layer associated with the collagen molecules. Release of these trapped water molecules could provide the increase of entropy required to drive the association of molecules into the fibrils.

Collagen structural models (Ramachandran and Chandrasekharan 1968; Ramachandran et al. 1973; Berg and Prockop 1973; Yee et al. 1974; Privalov et al. 1979) suggest that there are two types of intermolecular and intramolecular hydrogen bonds in fibrils: (I) a direct interchain hydrogen bond forms between the glycine residue and the residue in the second position of the neighboring chain, and (II) an additional hydrogen bond which links two adjacent tropocollagens using a bridging water molecule. This water-mediated hydrogen bonding makes two thirds of hydrogen bonds that connect neighboring peptides (Cameron et al. 2007) and therefore is a dominant interaction in stabilizing the fibrillar structure (Leikin et al. 1995; Kuznetsova et al. 1998). These water bridges are dynamically linked with freely exchangeable hydrogen atoms (Tourell and Momot 2016). Furthermore, water molecules can be confined by hydrophobic groups of neighboring tropocollagens (Hulmes et al. 1973) to maximize the number of water-water hydrogen bonds (Southall et al. 2002; Dill 1990). Since molecular assembly is driven by decreasing the number of unfulfilled hydrogen-binding opportunities at the protein-water interface (Fernández 2016), the trapped water molecules and the water bridges may have an important role in the collagen molecular assembly during fibril growth and remodeling (Martin et al. 2020).

3.2.3.2 Surface-Associated Proteoglycans

Proteoglycans are a superfamily of molecules distinguished by the covalent attachment of one or more highly negatively charged glycosaminoglycan chains to their core proteins (Comper and Laurent 1978), and they play a significant regulatory role during fibrillogenesis. Surface-associated proteoglycans and their glycosaminoglycan chains extend around the fibril and through steric effects limit lateral fibril

growth (Scott et al. 1981; Scott 1980, 1984; Scott and Orford 1981). A three phase model of fibrillogenesis and fiber maturation in rat tail tendon was proposed by Scott et al. (1981) In phase 1 (up to day 40 after conception), tropocollagen interacts with dermatan sulphate-rich proteoglycan during or immediately after formation of microfibrils. The hyaluronate and proteoglycan-rich environment and collagen synthesis increase the number of thin fibrils, rather than growth in diameter of established fibrils. In phase 2 (from day 40 to approximately day 120 after conception), concentrations of chondroitin sulphate-rich proteoglycan and hyaluronate decrease, promoting the addition of collagen to extant fibrils rather than formation of new fibrils, resulting in rapid increase of fibril diameter without axial periodicity change. In phase 3 (day 120 after conception onwards), fibril growth slows down and reaches its final structure.

Direct *in vivo* evidence for the role of proteoglycans in the regulation of collagen assembly and growth has been achieved by development of animals deficient in small leucine rich proteoglycans (SLRPs). The principal SLRPs found in tendon are decorin, biglycan, fibromodulin, and lumican. Both decorin and biglycan are expressed in the interfibrillar matrix and interfascicular matrix in postnatal development but they present distinct temporal patterns (Zamboulis et al. 2020; Zhang et al. 2006; Ansorge et al. 2012). Interfibrillar biglycan abundance in the mouse is highest early in development whereas decorin abundance peaks later during development; both are low in abundance at maturity (Zhang et al. 2006; Ansorge et al. 2012). Equine tendon shares the same temporal expression for decorin but biglycan abundance peaks later (Zamboulis et al. 2020). Both proteoglycans have a regulatory role in collagen fibril assembly during tendon development. Biglycan is believed to promote fibril diameter growth, whereas decorin is believed to control lateral fusion of the fibrils and increase fibril stability (Zhang et al. 2005). Decorin and biglycan-deficient mice show abnormal fibril structure and lateral fusion during development resulting in an increased number of small fibrils with a simultaneous presence of collagen fibrils with unusually larger diameter and decreased failure strength and stiffness once in maturity (Zhang et al. 2006; Ameye et al. 2002; Corsi et al. 2002). Decorin and biglycan also share a binding site for collagen type I (Schönherr et al. 1995) and an increase in biglycan abundance in decorin-deficient mice was observed, alluding to compensation between the two proteins (Zhang et al. 2006).

Both fibromodulin and lumican are found in the interfibrillar matrix of mouse tendon, with lumican expression peaking during early postnatal development and fibromodulin abundance peaking in the later stages (Ezura et al. 2000). In contrast, the temporal expression in the equine interfascicular matrix was reversed, with fibromodulin abundance early and lumican peaking towards the end (Zamboulis et al. 2020). Fibromodulin and lumican share a binding site on collagen type I implying that they are likely to have functional overlap (Svensson et al. 2000). Fibromodulin and lumican deficient and double deficient mice showed abnormal fibril structure, with lumican deficient mice displaying an increase in larger diameter fibrils and fibromodulin deficient mice an increase in smaller diameter fibrils at maturity. In the fibromodulin deficient mice, increased cross-linking of collagen was also observed (Kalamajski et al. 2014) and lumican expression was increased, suggesting compensation (Ezura et al. 2000). In the lumican deficient mice, the phenotype was less severe and tendon mechanical properties were not affected. Interestingly, the mechanical properties of double knockout mice were dependent on the number of functioning alleles pointing toward a regulatory role for fibromodulin and a modulatory role for lumican (Ezura et al. 2000; Jepsen et al. 2002).

Asporin and lubricin (PRG4) are also expressed in tendon interfibrillar and interfascicular matrix, but have received much less attention than the principal SLRPs. In developing equine tendon, asporin demonstrates a temporal pattern in the interfascicular matrix where it is increased in early development and subsequently decreases but remains present in mature tendon (Zamboulis et al. 2020; Henry et al. 2001; Peffers et al. 2015).

The role of asporin in tendon fibrillogenesis and mechanical properties has not been documented yet, but the skin of asporin deficient mice had increased expression of collagen type I and III, increased toughness, as well as a two-fold increase in decorin and biglycan levels (Maccarana et al. 2017). Lubricin, a large proteoglycan important for matrix lubrication (Rees et al. 2002; Kohrs et al. 2011; Sun et al. 2015a; Funakoshi et al. 2008; Nugent et al. 2006), is also found in the interfascicular matrix of equine tendon, with increasing abundance with development and in low abundance pericellularly in the interfibrillar matrix (Zamboulis et al. 2020). In lubricin deficient mice the gliding resistance of fascicles against each other was increased compared to null mice, confirming lubricin may play an important role in interfascicular lubrication (Kohrs et al. 2011). However, the role of lubricin in fibrillogenesis has not yet been elucidated in tendon.

3.2.3.3 pN-Collagen

There are several observations suggesting that N-propeptides are confined to the fibril surface (Watson et al. 1992; Holmes et al. 1991) where they block accretion of further molecules (Fleischmajer et al. 1981, 1983, 1985, 1987a, b; Nowack et al. 1976; Veis et al. 1973; Lapiere and Nusgens 1974; Timpl et al. 1975; Lenaers and Lapiere 1975). As a result, further lateral growth would be regulated by enzymic cleavage of the propeptides. The important role of N-propeptide has been observed in the studies of dermatosparaxis and Ehlers-Danlos syndrome (EDS) type VIIB. Dermatosparaxis is caused by partial loss of procollagen N-proteinase activity (Lapiere et al. 1971; Lenaers et al. 1971; Becker and Timpl 1976). Presence of N-propeptide on the surface of these fibrils results in a non-circular cross sections (Watson et al. 1998). Remarkably, it has been shown that dermatosparactic collagen fibrils will gain a normal appearance after implantation in normal animals (Shoshan et al. 1974), suggesting the existence of a dynamic mechanism for fibril growth and degradation. Also, Ehlers-Danlos syndrome type VIIB fibrils in which pN-collagen is only partially cleaved have rough-bordered and non-circular cross sections (Watson et al. 1992; Holmes et al. 1993).

Growth models (Hulmes 1983; Chapman 1989) have been proposed for collagen fibrils in which accretion of collagen molecules is inhibited by N-propeptides on the fibril surface. Growth of pN-collagen fibrils is inhibited due to the steric blocking of interaction sites by the N-propeptides. The growth inhibitor part of the molecules (the N-terminus) is confined to the fibril surface and the C-ends are buried inside the interior of the fibril. Since the growth inhibitors cannot act as a site for further accretion, their surface density increases with lateral growth. Growth of fibril diameter continues until fluidity in intermolecular contacts is restricted due to steric hindrance. This first critical diameter depends on the lateral width of the inhibitor segment, allowing for growth of fibrils with preferred diameters in different tissues (the inhibitor might vary in different tissues and stages of development, but the same mechanism still applies). When a fibril reaches uniformity at this critical diameter, accretion is limited to the fibril ends and growth is only in axial direction. Lateral growth can proceed to a second critical diameter after enzymatic removal of the growth inhibitor.

Romanic et al. (1991) in an *in vitro* study demonstrated that pN-collagen III can co-polymerize with collagen I, but cannot be deposited on previously assembled collagen I fibrils (Romanic et al. 1991). It was shown that the presence of pN-collagen III can (1) inhibit the rate of collagen I assembly, (2) decrease the amount of collagen I incorporated into fibrils, and (3) decrease the diameter of fibrils in comparison with fibrils generated under the same conditions from collagen I alone. Fibril diameter progressively decreased with increasing the initial molar ratio of pN-collagen III to collagen I. Therefore, it was concluded that pN-collagen III coats the surface of collagen I fibrils early in the process of fibril assembly and hinders lateral growth of the fibrils. But it does not bind to the growing tips of fibrils, resulting in formation of thin fibrils.

3.2.3.4 Minor Collagens

Other types of collagens are synthesized simultaneously with type I collagen (Gay et al. 1976; Burke et al. 1977; Foidart et al. 1980, 1983). The structural similarities of fibril forming collagens allow them to polymerize within the same "heterotypic" fibrils (Henkel and Glanville 1982; Fleischmajer et al. 1990). In tendon, approximately 95% of collagen is type I, with the remaining being mostly type III (Birch et al. 1999; Makisalo et al. 1989; Riley et al. 1994a; Amiel et al. 1984). Collagen type III is found both in the interfibrillar and interfascicular matrix of the developing tendon. In equine tendon, collagen type III expression increases throughout development in both the interfibrillar and interfascicular matrix reaching peak abundance towards the end of maturation (Zamboulis et al. 2020). Collagen type III distribution in the avian tendon is observed throughout the interfibrillar and interfascicular matrix early in development but solely in the interfascicular matrix later (Birk and Mayne 1997; Kuo et al. 2008). The decrease in collagen type III expression in avian tendon is also associated with the appearance of collagen fibrils with larger diameters implying participation of collagen type III in the regulation of collagen fibrillogenesis (Birk and Mayne 1997). Furthermore, collagen type III deficient mice demonstrated disrupted collagen fibrillogenesis and larger diameter fibrils, confirming the involvement of collagen type III in fibrillogenesis (Liu et al. 1997).

Collagen type V has also demonstrated a growth regulatory effect on collagen fibrillogenesis (Wenstrup et al. 2004; Birk et al. 1990a) and its mutations have been identified in patients with classic EDS (Malfait and De Paepe 2014; Symoens et al. 2012). Collagen type V is found in the interfibrillar and interfascicular matrix of the developing equine tendon and in the interfibrillar matrix of mouse tendon in association with the tenocyte surface (Zamboulis et al. 2020; Wenstrup et al. 2011; Smith et al. 2012, 2014; Sun et al. 2015b). Reduction of collagen V expression during development also results in formation of fibrils with larger diameters in other tissues such as the dermis (Wenstrup et al. 2006) and cornea (Segev et al. 2006). Corneal stroma, which contains collagen fibrils of uniformly small diameter (Hay and Revel 1969), is relatively rich in type V collagen with 20% type V to 80% type I (McLaughlin et al. 1989). Studies of type I/V interactions in the mature corneal stroma have shown that type I and type V collagen co-assemble into fibrils (Fitch et al. 1984; Birk et al. 1986, 1988; Linsenmayer et al. 1985, 1990) and decreasing the levels of type V collagen secreted by corneal fibroblasts *in situ* results in assembly of large-diameter fibrils with a broad size distribution (Marchant et al. 1996). *In vitro* fibrillogenesis studies (Birk et al. 1990a; Adachi and Hayashi 1986) also showed that fibrils produced from only type I collagen were thicker than hybrid fibrils of type I and type V collagen. In addition, collagen V-null mice tendons are smaller than their wild type counterparts and exhibit reduced mechanical function (Connizzo et al. 2015). However, the effect of collagen V deficiency on mechanical function is much more dramatic in joint stabilizing tendons and ligaments, suggesting a relationship between mechanical loading and collagen V mediated fibril development (Connizzo et al. 2015). Collagen type XI is found to be present early in development both in the mouse and equine interfibrillar matrix, and thought to play synergistic roles with collagen type V (Zamboulis et al. 2020; Wenstrup et al. 2011). Col11a1-null mouse models (Sun et al. 2020) show decreased body weights and their flexor digitorum longus tendon has abnormal collagenous matrix structure with a significant decrease in biomechanical properties. Absence of collagen type XI disrupts the parallel alignment of fibrils and increases fibril diameter, similar to collagen type V.

Collagen type XII and XIV are closely related members of the fibril-associated collagens with interrupted triple helices (FACIT) collagen class and have been identified in the interfibrillar matrix in mouse (Izu et al. 2020; Ansorge et al. 2009), and the interfibrillar and interfascicular matrix in the developing avian (Young et al. 2000; Zhang et al. 2003) and equine tendon (Zamboulis et al. 2020). Collagen type XIV levels are high in early development and decrease

thereafter to barely detectable levels in mature tendon whereas collagen type XII is more abundant in early development but also present throughout development, maturation, and aging (Zamboulis et al. 2020; Izu et al. 2020; Ansorge et al. 2009; Young et al. 2000; Zhang et al. 2003). Collagen type XII regulates lateral network formation and fiber domain compartmentalisation, as well as collagen type I secretion. Collagen type XIV plays a role in the early stages of tendon fibrillogenesis and entry into lateral growth, in accordance with its temporal expression. Absence of collagen type XII in Col12a1-null mouse model results in larger tendons with abnormal collagen fibril packing, increased stiffness, and decreased overall type I collagen (Izu et al. 2020). Also, type XIV collagen deficient mouse tendons demonstrate premature fibril growth and larger fibril diameters, but no deficiency in biomechanical properties at maturity (Ansorge et al. 2009). Despite being closely related, there does not appear to be a compensatory relationship in expression patterns (Izu et al. 2020; Ansorge et al. 2009).

Finally, collagen type VI has also been identified both in the interfibrillar matrix of developing mouse tendon, especially in the pericellular region, and in the interfibrillar and interfascicular matrix in equine developing tendon (Zamboulis et al. 2020; Smith et al. 2012; Izu et al. 2011). During development, collagen type VI was found to be implicated in maintaining the cell shape, microdomain structure and fiber organisation. Collagen VI deficient mice displayed abnormal fibril assembly in the pericellular region with more dense fibrils of smaller diameter and frequent very large or twisted fibrils (Izu et al. 2011). Other collagens such as collagen type IV and XXI show temporal expression in the development of the equine interfascicular matrix but they have received less attention and their role is not currently known.

3.2.3.5 Elastin, Fibrillins, and Fibulins

Elastin is found at the core of elastic fibers surrounded by a fibrillin-rich microfibril scaffold (Kielty et al. 2002). In tendon, its abundance is function-dependent, with a greater abundance of elastin found in energy storing tendons (Thorpe and Screen 2016; Godinho et al. 2017). Elastin is present during embryonic development and increases in response to mechanical loading (Oryan and Shoushtari 2008; Zamboulis et al. 2020; Wagenseil et al. 2010; Luo et al. 2018). Spatially, elastin is localized sparsely in the interfibrillar matrix parallel to the tendon axis and more densely in the interfascicular matrix, with both a parallel and perpendicular organization in relation to the tendon axis. Elastin haploinsufficiency in mice resulted in alterations to collagen fibril structure, favoring an increase in large diameter fibrils and reduced interfibrillar matrix, but these changes were site-specific (Eekhoff et al. 2017). The effect of elastin depletion on tissue function has also been debated, with some studies showing significant mechanical disruption and others demonstrating no effect (Eekhoff et al. 2017; Grant et al. 2015; Fang and Lake 2016). When fascicle and interfascicular matrix were interrogated separately following elastase treatment in equine tendons, fascicles did not show any changes in their mechanical properties. However, the interfascicular matrix was significantly compromised, suggesting a different role for interfibrillar and interfascicular elastin (Godinho et al. 2020).

Fibrillin-1 and 2 are known to be involved in elastogenesis and regulate activation and bioavailability of TGF-β superfamily members (Chaudhry et al. 2007; Boregowda et al. 2008). Fibrillin-1 and 2 are present in the interfibrillar and interfascicular matrix in mature tendon, colocalizing with elastin and also pericellularly on their own (Ritty et al. 2002; Kharaz et al. 2018). In developing equine tendon, fibrillin-1 and 2 were identified in the interfibrillar and interfascicular matrix with fibrillin-1 showing an increase in abundance during development in the interfascicular matrix only (Zamboulis et al. 2020). Fibrillin-1 deficiency in mice did not disrupt the tendon structure apart from generating smaller tendons (Tran et al. 2019) and fibrillin-2 deficiency resulted in a decrease in collagen crosslinking but did not affect tendon structure (Boregowda et al. 2008). It is possible that similar to elastin deficiency, the interfascicular matrix

is more profoundly affected by fibrillin-1 and 2 deficiencies than the fascicles.

Fibulin-4 and 5 are indispensable for elastogenesis (McLaughlin et al. 2006; Nakamura et al. 2002; Yanagisawa et al. 2002) and fibulin-4 is found in the tendon interfibrillar matrix co-localized with fibrillins (Markova et al. 2016). In fibulin-4 deficient mice, forelimb contractures were noted and collagen fibrillogenesis was disrupted in tendons (Markova et al. 2016). Fibulin-5 is found in the interfibrillar matrix but also the interfascicular matrix where its abundance in equine tendon peaks early in development (Zamboulis et al. 2020). In fibulin-5 deficient mice, malformed elastic fibers were found in tendon with no other changes to the composition or structure of the tendon. In addition, the linear modulus of the Achilles tendon was increased in the fibulin-5 deficient mice whereas the positional tibialis anterior tendon did not show any changes in mechanical properties (Eekhoff et al. 2021). Taken together, this supports a role for elastic fibers in the mechanical properties of functionally distinct tendons or tendon compartments beyond regulation of collagen fibrillogenesis.

3.2.3.6 Thrombospondins

Thrombospondins, specifically TSP-1, TSP-4, and COMP (TSP-5), have also recently been identified in the interfibrillar and interfascicular matrix of tendons (Kannus et al. 1998; Zamboulis et al. 2020; DiCesare et al. 1994; Hauser et al. 1995; Smith et al. 1997; Fang et al. 2000; Södersten et al. 2006; Havis et al. 2014; Schulz et al. 2016). COMP levels in the developing interfibrillar and interfascicular matrix increase with development and have been shown to be associated with loading (Zamboulis et al. 2020; Smith et al. 1997). In COMP deficient mice, the tendon structure exhibited larger fibril diameters with an increase in irregular shape, suggesting a role in collagen fibrillogenesis. In addition, collagen accumulation in the endoplasmic reticulum was detected in isolated dermal fibroblasts *in vitro*, alluding to its intracellular role in the secretion of collagen, which is dependent on the formation of a COMP-collagen complex (Schulz et al. 2016). TSP-4 has been reported to have a similar spatiotemporal expression as COMP, a function associated with loading, and also to be increased in COMP deficient mice (Schulz et al. 2016; Cingolani et al. 2011; Frolova et al. 2014). Similarly to COMP deficient mice, in TSP-4 deficient mice, tendons exhibited larger fibril diameters (Frolova et al. 2014). TSP-2 and TSP-3 have also been reported in the interfibrillar matrix of mouse tendon (Havis et al. 2014; Frolova et al. 2014; Kyriakides et al. 1998) and TSP-2 deficiency (Kyriakides et al. 1998) resulted in a similar collagen fibril phenotype noted in TSP-4 and COMP deficient mice (Schulz et al. 2016; Frolova et al. 2014).

3.3 Maintenance of the Matrix During Adulthood

3.3.1 Matrix Turnover

The pioneering studies of Schoenheimer and his collaborators in the 1930s changed the perception of proteins from a static collection of material to a material existing in a state of dynamic flux, where the balance of synthesis and degradation is critical to homeostatic maintenance of structure (Cohn 2002; Wilkinson 2018). The study of matrix turnover in maintaining adult tissue homeostasis, and the regulation of this process, has been the focus of much research over the past century since then and could be the key to preventing injury.

3.3.1.1 Collagenous Matrix

It is well established that collagen is one of the longest lived proteins in many tissues within the body, with a relatively low rate of turnover in skin, tendon and cartilage compared to other ECM proteins (Thorpe et al. 2010; Maroudas et al. 1998; Sivan et al. 2006, 2008; Verzijl et al. 2000). However, the specific rate of collagen turnover within tendon is still a matter of controversy, with conflicting data reported in the literature. Several studies have reported negligible turnover of tendon collagen within an individual's lifetime, with a half-life of 198 years in the

energy storing equine superficial digital flexor tendon determined by measuring the rate of aspartic acid racemization, and no collagen turnover detected in the healthy adult human Achilles tendon using ^{14}C bomb pulse data (Thorpe et al. 2010; Heinemeier et al. 2018, 2013a). However, other studies have reported relatively rapid collagen synthesis in tendon, with fractional synthesis rates of 0.04–0.06% hour^{-1} calculated in the human patellar tendon, which equates to half-lives ranging from 48 to 64 days (Miller et al. 2005; Babraj et al. 2005; Smeets et al. 2019). There are several potential explanations for these large discrepancies.

The studies in which high fractional synthesis rates were reported used stable isotope labelling over a very short time period, and it is unlikely that all newly synthesized collagen would be incorporated into the matrix, such that fractional synthesis rates would be overestimated. Indeed, using the tracer *cis*-[^{18}F]fluoro-proline combined with positron emission tomography and measuring protein incorporation in the rat Achilles tendon, it has been demonstrated that only approximately 20% of the proline taken up in the tissue was incorporated into the tendon matrix (Skovgaard et al. 2011).

The studies in which extremely long half-lives have been reported may also be affected by several factors. In these studies, the collagenous fraction of the matrix is purified using enzymatic digestion or protein extraction techniques (Thorpe et al. 2010; Heinemeier et al. 2018). Such purification techniques may result in some collagen loss; indeed approximately 13% of collagen was lost during purification by guanidine hydrochloride extraction (Thorpe et al. 2010). This is likely to represent more recently synthesized collagen that is less tightly cross-linked into the matrix, and therefore the half-life calculated based on the remaining collagen would be overestimated. There are also limitations associated with the methods used to estimate half-life; calculation of protein turnover rates using racemization of aspartic acid relies on assumptions made during calculations, as accumulation of D-Aspartic acid is affected by several factors, including temperature, pH, and protein structure (Thorpe et al. 2010). Precision of ^{14}C measurements is limited by variability in tissue radiocarbon levels within the population, which has progressively decreased over the past 50 years (Hodgins and U. S. Department of Justice 2009).

More recent studies also help to explain these previous contradictory findings, suggesting there may be pools of collagen within tendon that have differential turnover rates. Indeed, more collagen neopeptides, which are a marker of turnover, were identified within the interfascicular matrix compared to the fascicular matrix in the equine superficial digital flexor tendon (Thorpe et al. 2016a). These findings are supported by a recent study using *in vivo* isotope labelling combined with laser capture microdissection and mass spectrometry to measure the turnover rates of individual proteins within the fascicular and interfascicular matrices in the rat Achilles tendon (Choi et al. 2020). Results revealed significantly faster turnover of collagen in the interfascicular matrix compared to the fascicles, with a half-lives of 1.6 and 2.7 years for type I collagen in interfascicular matrix and fascicles respectively. While no studies have directly determined differences in turnover rates of extracellular matrix proteins between small and large animals, it is likely that protein turnover is more rapid in rodent models compared to humans, as previous studies have demonstrated a negative correlation between median protein turnover rate constants and lifespan (Swovick et al. 2018), and the half-life of serum albumin is approximately tenfold greater in the human compared to the rat (Chaudhury et al. 2003; Jeffay 1960).

Emerging evidence also suggests the presence of a sacrificial collagen matrix within tendon fascicles, with a recent study in murine tendon identifying the presence of thin collagen fibrils that are interspersed between thicker fibrils, and are synthesized and removed from the tendon within a 24 h period, while the bulk of the collagen remains unchanged (Chang et al. 2020). This rapidly turned over collagen may act to protect the long-lived collagen from mechanical damage, and also helps to explain previous studies which have measured both a high rate of synthesis, but very low rates of bulk turnover.

There is also evidence to suggest that collagen half-life varies between tendons with different functions, with a half-life of 198 years in the energy storing equine superficial digital flexor tendon compared to 34 years in the positional common digital extensor tendon (Thorpe 2010). While a lower rate of collagen turnover in high strain energy storing tendons may seem counter-intuitive, slower turnover may protect the tendon from remodeling which would weaken its structure, with the trade-off that when damage does occur it is more difficult to repair.

3.3.1.2 Non-collagenous Matrix

While only a small number of studies have measured rates of collagen turnover in tendon, even fewer have assessed turnover of non-collagenous proteins. It is, however, well established that non-collagenous protein turnover occurs at a more rapid rate than collagen turnover, with the exception of elastin, which is known to have very low turnover rate. While elastin half-life in tendon has not been measured, in other connective tissues there is compelling evidence that following development elastic fibers are not replaced throughout an individual's lifetime (Shapiro et al. 1991; Sherratt 2009). Aspartic acid racemization has been used to estimate turnover of the non-collagenous fraction of the extracellular matrix in functionally distinct equine tendons. However, this study was unable to provide turnover rates of individual proteins and a small amount of soluble collagen was detected in the fraction analysed, which is likely to affect the results (Thorpe et al. 2010). Despite these limitations, this study did show that turnover of non-collagenous proteins differed in tendons with different functions, with more rapid turnover in energy storing tendons compared to positional tendons (2.2 years vs. 3.5 years), which may allow for greater reparative capacity in injury-prone energy storing tendons (Thorpe et al. 2010).

Metabolism of different proteoglycan classes has been studied in tendon explants using radiolabelling, with results demonstrating relatively rapid turnover of newly synthesised large proteoglycans (half-life approx. 2 days) compared to small leucine rich proteoglycans (half-life approx. 20 days) and showing that different pathways are involved in the degradation of large and small proteoglycans (Samiric et al. 2004). However, this approach is only able to measure the turnover of newly synthesised proteoglycans rather than those already present within the matrix, which may be metabolized at a slower rate. More recent approaches using isotope labelling *in vivo* have measured turnover rates of a range of tendon proteoglycans, with half-lives ranging from 21 days for decorin to 72 days for lumican (Choi et al. 2020). There is also evidence to suggest that turnover rates of non-collagenous proteins may vary according to their location within the tendon matrix. Turnover of interfascicular decorin occurs at a faster rate than that of interfibrillar decorin (Choi et al. 2020). The reasons for this are unclear but indicate that proteoglycans may have distinct roles in different tendon regions.

3.3.2 Mechanical Stimulation for Matrix Remodeling

It is well established that mechanical stimulation drives the natural remodeling of the tendon ECM, and specifically the collagen structure (Zamboulis et al. 2020; Smith et al. 2002; Screen et al. 2005a; Batson et al. 2003; Bohm et al. 2015; Pan et al. 2018; Quigley et al. 2018; Theodossiou et al. 2019). Tenocytes can sense changes in their mechanical environment through cell-cell and cell-matrix interactions and transduce mechanical signals, which then trigger adaptive responses, a process called mechanohomeostasis (Fig. 3.4) (Maeda et al. 2012; Lavagnino et al. 2015; Heinemeier et al. 2003; Maeda et al. 2011; Havis et al. 2016). Since mechanotransduction pathways are comprehensively reviewed elsewhere (Wall et al. 2016, 2018; Humphrey et al. 2014), we report here on downstream changes in ECM structure in response to changes in mechanical stimuli. In addition, we focus on adaptations to normal loading and sub-failure damage rather than massive tissue injury/repair processes which

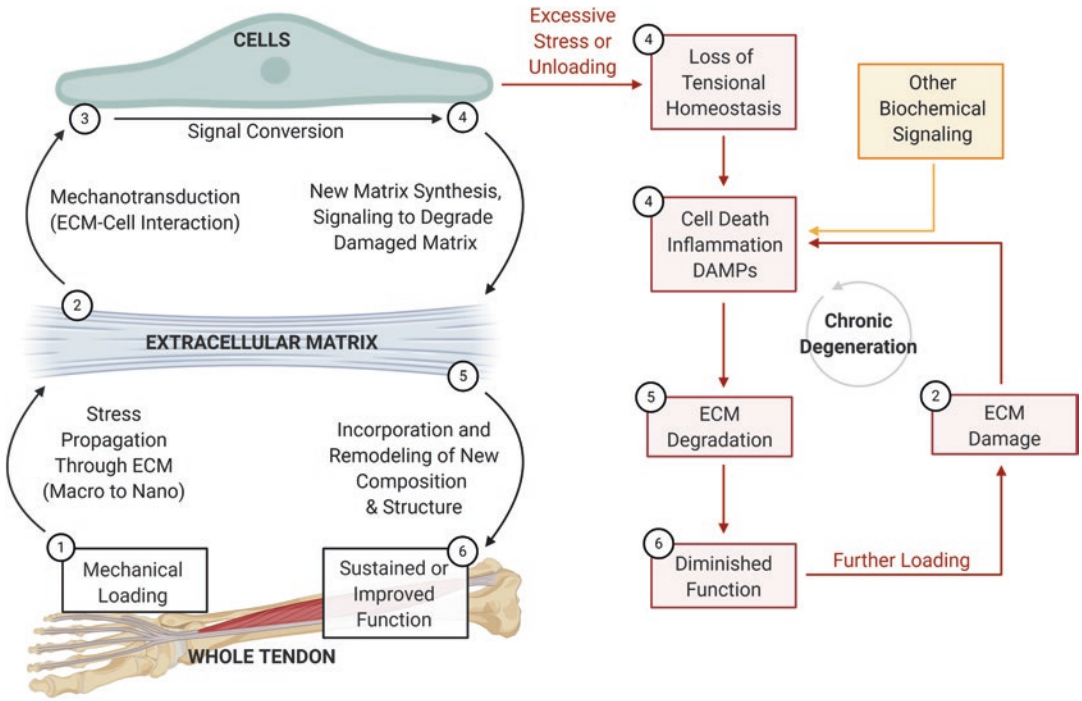

Fig. 3.4 Schematic of adult matrix mechanohomeostasis. (1) Multiaxial and multimodal mechanical loading on the tendon applies stress macroscopically to the tissue, which then (2) propagates through the multiscale hierarchy of the tendon matrix via interactions between the collagenous and non-collagenous matrix. Stress is then transduced from physical to biochemical signals in the cell via mechanotransduction (3), and these signals then trigger (4) catabolic or anabolic responses. In the case of normal loading or positive adaptation due to exercise (left), (5) new matrix is synthesized and incorporated into the existing structure while damaged matrix is removed resulting in (6) sustained or improved tissue function. In the case of excessive loading (overuse) or stress deprivation (disuse), there is a loss of tensional homeostasis at the cellular level which leads to the production of inflammatory markers and damage-associated molecular patterns (DAMPs) as well as increased matrix degradation and cell death (right). These signals can be spread to other cells through paracrine signaling, and can also be caused by other biochemical signaling or cellular changes (e.g., cell aging) in the absence of changes to mechanical loading (see Sect. 3.3.3). This process can lead to diminished function, and enter the tissue into a chronic degenerative cycle whereby further loading causes more matrix damage, eventually leading to tissue rupture and/or tendinopathy. (Created with BioRender.com)

are well described elsewhere (Thomopoulos et al. 2015; Andarawis-Puri et al. 2015; Andarawis-Puri and Flatow 2018).

3.3.2.1 Exercise

Alterations in mechanical stimuli can influence ECM turnover of adult tendons, with exercise and disuse both reported to result in a range of adaptations. However, the response seen in tendon is far less pronounced than that seen in muscle, and results are contradictory. In humans, there is evidence of tendon hypertrophy in response to exercise, with increases in patellar tendon cross sectional area (CSA) (Couppé et al. 2008; Farup et al. 2014). Studies have also reported increased markers of collagen synthesis and breakdown in peritendinous tissue both as a result of acute exercise and longer-term training in human Achilles and patellar tendons (Langberg et al. 1999, 2001; Astill et al. 2017). As collagen turnover rate in the tendon core is very low it has been suggested that additional newly synthesized collagen may be deposited around the edge of the tendon, resulting in increased CSA (Magnusson and Kjaer 2019). However, other studies which have taken tendon biopsies to assess collagen synthesis post-exercise in the patellar tendon report conflicting results, with some observing

increased collagen synthesis (Miller et al. 2005) and others reporting no change (Dideriksen et al. 2013; Hansen et al. 2009). This limited responsiveness is supported by studies which have either detected no, or very limited changes, in collagen and growth factor gene expression in response to exercise (Dideriksen et al. 2013; Heinemeier et al. 2013b; Sullivan et al. 2009).

These findings are in contrast to those reported in a variety of small animal models, which have demonstrated upregulation of tendon associated genes and increases in mechanical properties as a result of exercise or increased loading (Heinemeier et al. 2007, 2012; Olesen et al. 2006). However, the majority of small animal studies have been performed in animals that are not yet fully mature, such that they may have more capacity for adaptation to loading than skeletally mature human tendon. In addition, the type and duration of exercise performed is likely to influence results, with studies of the rat supraspinatus tendon demonstrating that a single bout of exercise tends to decrease mechanical properties, whereas chronic exercise results in improved mechanical properties (Rooney et al. 2017). This is accompanied by more matrix-related gene changes in chronic compared to acute exercise groups (Rooney et al. 2015). *Ex vivo* studies have also been performed to uncover the effects of loading on tendon metabolism, with mechanical loading of artificial tendon constructs i*n vitro* resulting in little change in tendon related genes at physiological levels of loading, but upregulation of genes associated with tendon development as a result of overloading (Herchenhan et al. 2020). By contrast, exposing fascicles from rat tail tendons to moderate degrees of loading increased collagen synthesis without generating mechanical or structural changes (Screen et al. 2005a; Legerlotz et al. 2013b).

3.3.2.2 Disuse or Stress Deprivation

Disuse has been shown to result in a marked decline in tendon mechanical properties, both in humans and animal models (Magnusson and Kjaer 2019; Rumian et al. 2009; Almeida-Silveira et al. 2000; Matsumoto et al. 2003; Couppé et al. 2012). However, the mechanisms by which these alterations occur are unclear, as the majority of studies do not report any alterations in tendon dimensions or mass as a result of unloading (Kinugasa et al. 2010; de Boer et al. 2007; Heinemeier et al. 2009). Some studies have reported decreased patellar tendon collagen synthesis as a result of lower limb suspension in the human, even after relatively short periods of disuse (de Boer et al. 2007; Dideriksen et al. 2017), accompanied by increased matrix metalloproteinase 2 (MMP-2) expression (Boesen et al. 2013). By contrast, results from animal studies are variable and sometimes contradictory; hind limb suspension in the rat resulted in very few alterations in the Achilles tendon (Heinemeier et al. 2009), whereas denervation-induced unloading of the mouse patellar tendon caused decreased expression of type I collagen, increased expression of MMP-13 and a decrease in collagen fibril diameter (Mori et al. 2007). Explant models have been used to further investigate the effect of unloading on tendon metabolism, with stress deprivation of murine tail tendon fascicles resulting in increased levels of matrix degrading enzymes and reduced mechanical properties (Abreu et al. 2008; Lavagnino et al. 2003, 2005; Wunderli et al. 2018). More recent studies demonstrate decreased expression of genes associated with both matrix synthesis and degradation in stress deprived murine flexor tendons (Connizzo et al. 2019).

The contradictory findings from animal studies may be due to differences in species and ages in studies, the particular model of mechanical stimulation employed, and also whether the experiments have been performed *in vivo* or *ex vivo*. In addition, it has been reported that functionally distinct tendons also display a differential response to unloading *ex vivo*, with more rapid and extensive changes seen in positional compared to energy storing tendons (Choi et al. 2019). Further, stress deprivation may preferentially affect the interfascicular matrix, with greater deterioration in this region compared to the fascicles in unloaded rat tail tendon (Rowson et al. 2016). Different types of mechanical stimulation can also generate different responses and, *in vitro*, tenocytes are mostly stimulated using

tension, which likely mirrors the mechanical stimulation interfibrillar tenocytes experience *in vivo*. However, *in vitro* shear stress stimulation of adult tenocytes, which is likely experienced by interfascicular tenocytes, generated an "anti-fibrotic" expression pattern with decreased transcription of collagen type I and III (Fong et al. 2005). In addition, the responses to mechanical stimulation may also be influenced by the age of the cells or tissues *in vitro (*Zamboulis et al. 2020*;* Fong et al. 2005*)* and the magnitude of loading (Zhang and Wang 2013).

3.3.2.3 Sub-failure Microdamage

In addition to normal exercise, studies have sought to understand the capacity for intrinsic repair of microdamage that occurs due to tendon overload. Several *in vivo* models have been developed to induce tendon fatigue damage, including treadmill running and repetitive reaching activities (Glazebrook et al. 2008; Carpenter et al. 1998; Gao et al. 2013). A model developed by Fung et al. (2010) in which the rat patellar tendon is clamped and loaded directly while the animal is under anaesthesia allows precise loads to be applied to the tendon, while the number of cycles applied can be varied to induce different degrees of damage. This model has been used to extensively characterise the structural, mechanical and molecular changes within tendon to varying levels of fatigue damage at different time points. Results show that structural alterations become more pronounced as severity of fatigue loading progresses, with isolated collagen fiber kinking in response to low-level fatigue loading which becomes more widespread in moderate fatigue loading and is accompanied by fiber separation. Severely fatigue loaded tendons exhibit widespread matrix disruption and fiber thinning (Fung et al. 2010). These structural changes are associated with alterations in mechanical properties, with a single bout of moderate fatigue loading being sufficient to induce accumulation of structural damage associated with non-recoverable loss of stiffness (Bell et al. 2018). These studies indicate a limited ability for intrinsic repair of damage above a certain threshold, even when no further loading is applied.

Considering the molecular changes as a result of fatigue loading, expression of genes associated with matrix remodeling, including collagens and MMPs, were negatively correlated with the degree of damage (Andarawis-Puri et al. 2012), suggesting an impaired ability to repair microdamage as the damage worsens. In addition, apoptosis within the tendon increased with damage (Andarawis-Puri et al. 2014), likely due to alterations in cell microenvironment. Increased apoptosis will likely decrease the capacity for matrix remodeling, leading to further damage accumulation. The authors of these studies suggest that restoration of cell microenvironment may be key to improving the capacity of resident tendon cells to successfully remodel regions of microdamage (Andarawis-Puri and Flatow 2018). Exercise performed post-fatigue loading provides a method of influencing cell microenvironment and subsequently matrix synthesis, and, depending on timing, can either worsen or improve repair. Exercise initiated 2 weeks after fatigue loading resulted in increased levels of procollagen-I, indicative of matrix remodeling, whereas exercise that commenced immediately after fatigue loading caused further damage to the tendon, accompanied by increased levels of aggrecan and collagen type III, proteins that are both associated with a failed healing response (Bell et al. 2015). It is likely that post-fatigue loading exercise also influences matrix degradation, however this is yet to be directly determined.

There is also emerging evidence to suggest that initial overload damage within the tendon may occur within the interfascicular matrix. In bovine and equine flexor tendon explants exposed to cyclic loading *in vitro*, initial damage occurred preferentially to the interfascicular matrix, with upregulation of inflammatory mediators observed in this region (Spiesz et al. 2015; Thorpe et al. 2015b). The high shear environment within the interfascicular matrix of energy storing tendons, caused by interfascicular sliding as the tendons stretch, is likely to expose the resident cells to a complex strain environment incorporating tension, shear and compression (Cook and Screen 2018). Overload may therefore induce cell-

mediated degradation, and subsequent loss of interfascicular matrix structure is likely to alter cell microenvironment within the fascicles, leading to propagation of damage throughout the tissue. However, the majority of rodent tendons lack an interfascicular matrix structure (Liu et al. 2016; Lee and Elliott 2019), and therefore the response of the interfascicular matrix to microdamage cannot be studied using these models, limiting our knowledge in this area.

3.3.3 Biochemical Disruption of Matrix Homeostasis

There are a variety of biochemical stimulators that can influence tendon homeostasis. While inflammation occurs in the initial response to tendon injury, inflammatory mediators, including prostaglandins and cytokines, are also upregulated in tendon in response to exercise (Langberg et al. 1999, 2002). Blocking prostaglandin E_2 (PGE_2) by administration of non-steroidal anti-inflammatories resulted in decreased peritendinous collagen synthesis in response to exercise in the human patellar tendon, and collagenase upregulation in rat tendon cells (Christensen et al. 2011; Tsai et al. 2010). In addition, peritendinous infusion of (interleukin-6) IL-6 elevates collagen synthesis in a similar manner to exercise (Andersen et al. 2011). Inflammatory mediators also influence proteolytic activity, with IL-1β acting in synergy with mechanical stretch to increase levels of matrix degrading enzymes in rabbit tendon fibroblasts and human patellar tendon derived cells (Archambault et al. 2002; Yang et al. 2005). Recent studies also show that IL-1 and IL-6 can directly lead to matrix degeneration using an *in vitro* model system (Connizzo and Grodzinsky 2018b, 2020). Collectively, these results suggest that inflammatory mediators are important stimulators of collagen turnover in tendon that can act independently of loading. However, it is likely that only a very small proportion of newly synthesized collagen is incorporated into the matrix, and therefore upregulation of matrix metalloproteases does not necessarily alter tendon mechanical properties or collagen content (Marsolais et al. 2007). Interestingly, it seems that regular mechanical loading is required to protect rat tail tendons cultured in the presence of inflammatory cells from degradation and loss of mechanical properties (Marsolais et al. 2007), highlighting the importance of mechanical stimuli for maintenance of tendon homeostasis.

Systemic diseases can also affect tendon metabolism and increase the risk of tendon injury. Diabetes is associated with increased prevalence of tendinopathy and disorganization of the collagen fibers within human tendon (Abate et al. 2013). Tendons from diabetic mice have smaller cross-sectional areas, reduced mechanical properties and altered collagen fiber alignment, and these alterations vary between tendon types (Connizzo et al. 2014b). It is hypothesized that these changes are caused by the accumulation of advanced glycation end-products (AGEs) due to the increased availability of glucose, causing loss of both biological and mechanical function (Abate et al. 2013). These AGEs are also known to accumulate naturally during the aging process. Studies have also shown that treating rat tendon-derived cells with high glucose results in down-regulation of ECM-associated genes (Wu et al. 2017), indicating alterations in tendon homeostasis.

Obesity is another recognized risk factor for tendon injury, initially postulated to be caused by the increased mechanical strain due to weight. However, it has recently been established that adipose tissue is a potent releaser of signaling molecules, with raised serum levels of inflammatory markers present in obese individuals suggesting the presence of low grade inflammation, which could disrupt tendon homeostasis (Abate et al. 2013; Cilli et al. 2004). Indeed, in diabetic and obese mice, collagen and MMP expression is elevated during tendon healing, with increased macrophages and delayed remodelling (Ackerman et al. 2017a). Other metabolic disorders are also associated with tendon pathologies, including hypercholesterolemia, which results in cholesterol deposits in tendon, accompanied by alterations in tenocyte gene and protein expression, matrix turnover, tissue vascularity, and cytokine production (Soslowsky and Fryhofer

2016). It appears these disorders all affect tendon homeostasis via a variety of mechanisms which often involve inflammatory mediators, resulting in altered turnover and disruptions to the tendon matrix leading to increased risk of pathology.

3.3.4 Circadian Regulation

Recent studies have also unveiled the importance of the circadian clock in regulating tendon protein turnover, with rhythmic expression of several clock-associated genes resulting in nocturnal procollagen synthesis and diurnal fibril assembly in mice. This pool of newly synthesized collagen is then rhythmically degraded. This could be a primary mechanism for repairing microdamage that accumulates over a single day of use, but the incorporation of these newly synthesized collagen fragments into the existing matrix has not yet been confirmed. Disabling the circadian clock results in formation of abnormal collagen fibrils and collagen accumulation, indicating that protein homeostasis in tendon is maintained by circadian regulation of a sacrificial collagen matrix (Chang et al. 2020). While endogenous circadian rhythms have been observed in human tendon cells, studies have not yet been able to detect any alterations in expression of clock-associated genes within tendon as a result of exercise or immobilization (Yeung and Kadler 2019; Yeung et al. 2014). However, expression levels of clock genes in these studies were very low, and there were high levels of variability between individuals. Therefore, more studies are needed to determine if the alterations in tendon turnover as a result of changes to loading environment occur via circadian regulation.

3.4 Dysregulation of ECM Structure and Function During Aging

Aging is one of the primary risk factors for degenerative tendon injuries, particularly in the Achilles tendon and the rotator cuff tendons (Wertz et al. 2013; Strocchi et al. 1991; Minagawa et al. 2013; Longo et al. 2011; May and Garmel 2020). These injuries cause significant pain, frailty and a loss of independence, leading to a general reduction in quality of life (Kjær et al. 2020). Age-related disorders are associated with a degenerative tendon state, rather than an acute tendon rupture, which is thought to be a result of repetitive damage to the extracellular matrix (Fig. 3.4). However, the ability to study tendon aging in a controlled and repeatable fashion is quite challenging. Results are heavily dependent on the tendon being studied, the methodology used and on the ages defined as ‘young’ and ‘old’. Furthermore, aging is a complex and multifactorial process, involving natural changes in structure and function as well as alterations to the biological processes that regulate tissue architecture.

3.4.1 Changes to Matrix Structure and Function with Age

Historically, aging studies in tendon have focused on alterations in tendon structure and function in aged individuals, and in particular on detecting changes in the collagenous structure. However, findings of age-related changes in collagen morphology appear to be species- and tendon-dependent. Collagen content has been shown to increase (Stammers et al. 2020), decrease (Couppé et al. 2009; Sugiyama et al. 2019), or remain unchanged (Birch et al. 1999; Thorpe et al. 2010; Kostrominova and Brooks 2013) with increasing age in a variety of model systems, despite also reporting downregulation of collagen mRNA expression (Kostrominova and Brooks 2013). Equine research shows a decrease in tendon fibril diameters with increasing age (Parry et al. 1978a), hypothesized to lead to increased fibrillar interaction and reduced interfibrillar sliding (Ribitsch et al. 2020). Alterations in collagen cross-linking are also debated in the literature, with increases in mature cross-links observed in old human subjects (Couppé et al. 2009) while overall cross-linking levels decreased with age in mouse tail tendon fascicles (Stammers et al. 2020). However, non-enzymatic crosslink-

ing associated with advanced glycation end-products was increased in both studies.

Studies in age-related alterations in the structural organization of the collagenous and non-collagenous tissue compartments are similarly inconclusive. Studies in rat tail fascicles using polarized Raman spectroscopy demonstrate changes in collagen fiber orientation with aging, specifically indicating a more homogeneous tissue structure (Van Gulick et al. 2019), yet histological studies report disruption of collagen fiber organization in aged mouse tendons (Sugiyama et al. 2019). Other studies have also demonstrated altered crimp morphology in the flexor tendon of older horses (Patterson-Kane et al. 1997). Crimp frequency and amplitude in the murine flexor and patellar tendons were no different with age, but the change in crimp amplitude in response to mechanical loading was larger in older flexor tendons (Zuskov et al. 2020). Interestingly, the number of collagen fascicles was observed to decrease with age, suggesting a shift towards a greater proportion of interfascicular matrix in older tendons (Ali et al. 2018; Gillis et al. 1997).

Similar to age-associated changes in collagen content, inconsistent differences in glycosaminoglycan (GAG) levels have been observed. GAG content is decreased with age in the human supraspinatus tendon, but not in the biceps tendon (Riley et al. 1994b). However, GAG content was no different in male or female murine flexor tendons (Connizzo et al. 2019). Research in the equine model showed tendon-specific changes in GAG content, with age-associated decreases in positional tendons but no difference in energy storing tendons (Thorpe et al. 2010). This alludes that changes in GAG content with aging may be specific not only to the tendon studied but also perhaps to regional differences within the tendon. For example, accumulation of GAGs has been reported in tendinopathy samples, which is highly associated with aging, and tendons with regions that wrap around bone such as the rotator cuff and the insertion of the Achilles tendon (Thornton and Hart 2011; Archambault et al. 2007; Attia et al. 2012; Majima et al. 2000).

With respect to the other non-collagenous components of tendon, there are few studies investigating age-related changes. Measures of DNA content, and therefore tissue cellularity, do not change in aged equine tendons (Birch et al. 1999). One recent study in aged murine flexor tendons demonstrated a significant reduction in cell density in aged murine flexor tendons, but this change appeared to be sex-dependent with no differences found in age-matched female tendons (Connizzo et al. 2019). Cell density has also been shown to decrease in both rabbits and rats (Magnusson and Kjaer 2019; Nakagawa et al. 1994). In addition to cell number, tenocyte shape has also been reported to be altered in aging, with a shift towards a higher nucleus to cytoplasm ratio and a reduction of other organelles (Ippolito et al. 1980). Elastic fibers, typically found between collagen fibers and fascicles, have been reported to decrease and become more disorganized during aging (Godinho et al. 2017; Eekhoff et al. 2017; Ippolito et al. 1980), potentially altering sliding and stretch mechanisms at the microscale. Lubricin, which acts as a lubricant to enable gliding function (Funakoshi et al. 2008; Sun et al. 2006; Taguchi et al. 2009), has been reported to increase with age in rabbit tendons (Thornton et al. 2015) but remain unchanged in human Achilles tendon (Peffers et al. 2015). Finally, aged mice have been reported to have increased calcification, reduced vascularization, and increased adipose tissue (Zhang and Wang 2015; Marqueti et al. 2017).

Changes in tissue structure do not appear to translate into clear deficits in macroscopic tissue function. In fact, age-related changes in quasi-static mechanical properties appear to vary based on the specific tendon studied, the protocol used to assess changes, and the boundary conditions (gripping, testing environment, etc.) for experimentation (Ackerman et al. 2017b; Vogel 1980; Shadwick 1990; Haut et al. 1992). Tendon mechanical properties have shown to both decrease (Vogel 1980) and increase with age in rat tail tendons (Shadwick 1990; Nielsen et al. 1998). In rat patellar tendon, mechanical properties were weakly positively correlated with age (Haut et al. 1992) or decreased with age (Dressler et al. 2002). Achilles tendon function is decreased in older humans (Lindemann et al. 2020), and

either decreased (Pardes et al. 2017) or no different (Gordon et al. 2015) in aged rodents compared to mature counterparts. Rotator cuff tendons do not appear to have altered macroscale function with aging (Connizzo et al. 2013b; Lin et al. 2020). Interestingly, measures of dynamic tissue function through fatigue loading (Zuskov et al. 2020; Thorpe et al. 2017), dynamic macroscopic testing (Pardes et al. 2017; Dunkman et al. 2013), and measures of dynamic responses at the fiber (Connizzo et al. 2013b; Li et al. 2013) and fibril (Thorpe et al. 2013b) levels all suggest a diminished mechanical function in the aging population. In addition, nanomechanical testing revealed increased fluid flow and poroelasticity in aged supraspinatus tendons but decreased compressive function (Connizzo and Grodzinsky 2018a), alluding to deficits in dynamic mechanical function. These dynamic and nanoscale evaluations are indicative of changes present in the extracellular matrix, but could be more associated with changes in the interfibrillar or interfascicular matrix (Thorpe et al. 2013b, 2015a, 2017) rather than the collagenous matrix.

3.4.2 Matrix Turnover in Aged Tendons

Like many other tissues, it has been well established that the matrix repair response in aged tendons is impaired (Ackerman et al. 2017b; Mienaltowski et al. 2016). Recent studies have focused primarily on massive injury responses as a result of partial or full-thickness tendon tears. However, we focus here on the ability of aged cells to regulate everyday tissue homeostasis. Although tendons typically are thought to have very low matrix turnover at maturity, tenocytes do become metabolically active, begin to proliferate and actively remodel the matrix in response to changes in mechanical stimulus (Heinemeier et al. 2012; Rooney et al. 2014, 2015; Magnusson and Kjaer 2003; Kjaer et al. 2005). As reported here and in studies before, only a small fraction of the collagen present in tendons, hypothesized to be associated with small diameter collagen fibrils, is homeostatically regulated for daily remodeling to comply with functional demands (Chang et al. 2020; Thorpe et al. 2010; Yeung and Kadler 2019; Birch et al. 2016). However, the turnover rate of this small fraction has not been studied extensively in aged tendons to date (Birch et al. 2016). One study in equine tendons suggested that there is a decline in collagen turnover in aged tendons, while other studies of diseased tendon show increased collagen turnover rate (de Mos et al. 2007). Recent investigations of collagen synthetic activity in horse tendons reported no differences though (Thorpe et al. 2015b), suggesting no difference in the capacity to remodel the matrix. However, recent studies in mouse tendon explants demonstrated that although there were no differences between young and aged mice synthetic activity at baseline, age-related declines were evident when subjected to stress deprivation (Connizzo et al. 2019). Perhaps an injurious stimulus is necessary to illuminate larger deficits in matrix synthesis due to the generally low metabolic activity of tendon *in vivo*, and this highlights potential deficits that could be present in homeostatic remodeling and tissue repair but are not yet explored.

The interfascicular matrix has recently been shown to contain more proteins and more protein fragments than the collagenous compartment, indicating greater matrix degradation and turnover (Thorpe et al. 2016a). Since dynamic reorganizations such as collagen sliding and re-alignment are responsible for much of the daily function of tendons, the interfascicular compartment is likely more often damaged and remodeled accordingly. In fact, the interfascicular matrix, and not the fibrous matrix, was recently shown to be the primary location of adaptation to mechanical loading during development, highlighting the importance of this compartment in understanding overall tissue turnover (Zamboulis et al. 2020). While protein quantity does not change with aging in the interfascicular matrix, the number of protein fragments decreased indicating decreased matrix turnover and accumulation of tissue damage, potentially leading to chronic disease (Thorpe et al. 2016a).

3.4.3 Aging-Associated Changes in Cell Function Affecting Matrix Homeostasis

One difficulty in identifying mechanisms for age-related tissue degeneration is the inability to disentangle changes in the ECM and changes in cell behavior. Since resident cell populations are critical to maintaining and repairing the extracellular matrix in mechano-homeostasis (Fig. 3.4), it is likely that changes with age in the extracellular matrix are preceded by cellular adaptations. Age-related cellular changes have been characterized extensively in other organ systems, defined as nine primary hallmarks of aging (López-Otín et al. 2013; Hernandez-Segura et al. 2018). This includes genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. The first four hallmarks represent primary causes of cellular damage, while the other five are either responses to that damage, initially attempting to mitigate the damage but eventually becoming damaging themselves, or consequences of that damage. Since there are several other reviews that describe these hallmarks and their effects on cell behavior extensively (López-Otín et al. 2013; Folgueras 2018; Guerville et al. 2020; Rebelo-Marques et al. 2018), we focus here on those changes that may be relevant to understanding age-related changes in matrix turnover, based on literature in the tendon and ligament field as well as studies performed in other fibrous tissues.

3.4.3.1 DNA Damage and Matrix Turnover

Although we have DNA repair mechanisms, damage naturally accumulates over time via exposure to environmental toxins, simple DNA replication errors, or damage molecules. Telomeres protect the terminal ends of chromosomes from deterioration, but since cells are not able to copy the ends of DNA efficiently the telomere region shortens with each cell division. After some time, this can lead to cell growth arrest, limiting the ability of tissues to grow and regenerate with aging. Stem cells harvested from the periodontal ligament were reported to have significantly shorter telomere length with increasing donor age, and this corresponded with reduced regenerative properties (Ng et al. 2020; Trivanović et al. 2015). In contrast, relative telomere length was not decreased in aged equine tendons (Thorpe et al. 2016b). Given differences in collagen turnover rate between the two tissues, with periodontal ligament being associated with much faster turnover, DNA damage accumulation due to telomere shortening may be dependent on specific tendon function. Since tenocytes more generally have a fairly low proliferation rate at maturity (Grinstein et al. 2019), it is unclear what role replication-based damage would play in tenocyte behavior; it is more likely that these mechanisms would impact tendon-derived stem or progenitor cells (Kohler et al. 2013).

Proteins are constantly being synthesized and degraded throughout our lifetime to maintain an efficient and effective functional tissue. The regulation of protein assembly inside the cell, an array of quality control mechanisms, is called protein homeostasis or proteostasis (López-Otín et al. 2013; Klaips et al. 2018). These mechanisms become less efficient in aged organisms, which can result in protein aggregation as well as the production of damaged or misfolded proteins which can cause cell and tissue dysfunction. Decreased expression of genes encoding molecular chaperones facilitating protein folding and proteostasis has been reported in fibroblasts harvested from skin in patients with classic EDS (Chiarelli et al. 2019a, b). Given the tendon and ligament phenotype in this disease, this work provides evidence that loss or inefficient proteostasis could be a mechanism for disrupted matrix production in fibrous tissues. Furthermore, another recent study reported interplay between collagen synthesis and endoplasmic reticulum stress via the circadian clock, whereby targeting protein misfolding in disease could restore collagen homeostasis (Pickard et al. 2019). Future work is necessary to determine if loss of proteos-

tasis is an age-related phenomenon in tenocytes or tendon stem cells and what direct effects this might have on matrix homeostasis.

3.4.3.2 Mitochondrial Dysfunction and Oxidative Stress

A byproduct of mitochondrial energy production is the presence of free radicals or reactive oxygen species (ROS), which can be potentially damaging to the cell. For many years, ROS were thought to be the major culprit behind aging but recent studies showing that lowering ROS does not impact health have challenged this idea (López-Otín et al. 2013; Hekimi et al. 2011; Van Remmen et al. 2003). Production of ROS is important for signaling cell stress, but this process is a delicate balance. Over time, increasing production of ROS results in dysfunction of the mitochondria which in turn can lead to cells becoming less efficient at producing energy and causing damage to other cellular components (López-Otín et al. 2013). One recent study reported increases in the expression of peroxiredoxin, an antioxidant, in degenerated tendon, suggesting that oxidative stress may be a factor in the etiology or progression of age-related tendon disease (Wang et al. 2001). In addition, a reduction in catalase and heat shock proteins discovered through proteomic analysis suggests that aged tendons may be prone to ROS-based damage (Peffers et al. 2014). However, other studies have found no changes in oxidative-stress related genes (Peffers et al. 2015). Several studies have suggested that DNA damage in tendon cells can be induced through the production of ROS via mechanical overload or underload (Yudoh et al. 2005; Zapp et al. 2020). In fact, repression of oxidative stress through drug therapies diminishes the aberrant differentiation of tendon-derived cells subjected to excessive mechanical overload (Hsiao et al. 2019; Morikawa et al. 2014). Physiological loading was found to reduce the production of oxidative products such as ROS, protecting cells from premature senescence and matrix degeneration (Zhang and Wang 2015).

Besides the study of reactive oxygen species produced in the mitochondria, there have not been many studies on the role of mitochondrial function in tendon aging more generally. One recent study found that treating rat-derived tendon fibroblasts with advanced glycation end-products caused alterations in mitochondrial DNA content as well as a shift in matrix remodeling towards degradation rather than synthesis (Patel et al. 2019). Mitochondrial biomarkers are upregulated in the early phases of tendon healing, and therefore dysfunction in this organelle may also play a role in impaired tissue healing found in aged individuals (Thankam et al. 2018). Furthermore, the export of mitochondrial calcium is a key process in the process of matrix calcification during tendon calcification, suggesting a link with matrix production (Yue et al. 2016). Given these links between mitochondrial function and matrix production, clearly more research into the role of mitochondrial function in normal tenocyte or tendon stem cell homeostasis is warranted.

3.4.3.3 Cellular Senescence and SASP in Matrix Degradation

Cellular senescence is a natural repair response to damage, which can arise due to overexpression of certain oncogenes, by excessive cell replication, or by the presence of certain DNA damage-causing molecules (Acosta et al. 2013; Blagosklonny 2011; Blokland et al. 2020). Senescence is critical for wound repair and tumor suppression in young and mature individuals, preventing damaged cells from continuing to proliferate and propagate throughout the tissue. However, in old tissues, clearance of these cells is deficient, likely due to deteriorating immune function and thus, senescent cells accumulate within the matrix. One major concern with the presence of senescent cells is their ability to produce pro-inflammatory cytokines, called the senescence-associated secretory phenotype (SASP) (Miller et al. 2012b; Connizzo et al. 2013b). Inflammatory signaling produces many deleterious effects on matrix metabolism, as discussed above in Sect. 3.3.3 (Acosta et al. 2013; Zhang et al. 2015; Tsuzaki et al. 2003; Fedorczyk et al. 2010). Therefore, exposure to high levels of inflammatory cytokines may tip the scales towards matrix degeneration over adaptation

(Connizzo and Grodzinsky 2018b, 2020). Importantly, the SASP reinforces senescence through paracrine signaling (Acosta et al. 2013), thus a small population of senescent cells in aged tissues can lead to significant declines in tissue maintenance (Campisi 1998). This inflammatory signaling can also be further stimulated by damage-associated molecules produced in the extracellular matrix regularly, such as soluble decorin, tenascin-c and fibrinogen (Blokland et al. 2020).

Both tenocytes and tendon stem cells (TSCs) have been induced to replicative senescence *in vitro*, and cells harvested from aged subjects have been shown to favor senescence induction earlier than young counterparts (Kohler et al. 2013; Arnesen and Lawson 2006). Cells prematurely induced to senescence in the laboratory have been critical at understanding the age-related process, but cell culture alone does not replicate native cell-cell and cell-matrix connections for studying ECM remodeling. Therefore, the link between cellular senescence and dysregulation of ECM maintenance has not yet been fully elucidated. There does appear to be a connection between matrix synthesis and cellular senescence. One study demonstrated that collagen I is upregulated in senescent fibroblasts harvested from human subjects and in cells subjected to hydrogen peroxide to induce senescence (Murano et al. 1991; Dumont et al. 2000), indicating a role for collagen production in senescence. Interestingly, research using senescence-accelerated mouse models demonstrated that senescence-prone cells respond to collagenase-injection with altered expression profiles favoring matrix degradation over synthesis (Ueda et al. 2019). In fact, increased expression of MMPs has been reported before generally with aging and also specifically in aging tendon and senescent cells (Dudhia et al. 2007; Jones et al. 2006; Yu et al. 2013; Millis et al. 1992). In the absence of mechanical signals (as in the case of disuse or injury), aged mouse flexor explants exhibited increased expression of MMPs and cellular senescence markers (p16/p19/p53) (Connizzo et al. 2019). Therefore, there does appear to be a relationship between senescence and collagen turnover although it is unclear whether collagen is typically increased or decreased due to discrepancies between studies. Senescence has been implicated in fibrosis of the lung and in cutaneous wounds (Waters et al. 2018; Jun and Lau 2017), but further work is needed to clarify this link in tendon and ligament tissues.

3.4.3.4 Tendon Stem Cell Exhaustion and Matrix Repair

Like other cells, stem cells are also subject to age-related changes such as DNA damage accumulation, telomere shortening and cellular senescence. Over time, these lead to changes in the behavior of the stem cells present as well as a reduction in the pool of stem cells available. Age-related changes in TSCs is one of the more commonly studied mechanisms of aging in the tendon and ligament literature (Lui and Wong 2019; Zhou et al. 2010; Dai et al. 2019), thought to be a primary mechanism for age-related declines in tendon healing. TSCs are present in lower numbers in aged rabbit (Zhang and Wang 2010), rat (Zhou et al. 2010), and human tendons (Kohler et al. 2013; Ruzzini et al. 2014). Since these cells are often recruited to injury sites to aid in tissue repair, this reduction in cell number is hypothesized to be a primary determinant of diminished healing capacity.

While multiple studies have also shown that the self-renewal capacity of TSCs is not altered with aging (Kohler et al. 2013; Zhou et al. 2010; Ruzzini et al. 2014), the functional capacity of these cells to perform duties necessary for matrix remodeling and repair is indeed altered. Aged TSCs exhibit lower proliferative capacity and reduced migration (Zhang and Wang 2015; Kohler et al. 2013; Zhou et al. 2010), suggesting insufficiencies in recruitment of TSCs to repair sites in aged tendons. However, the recruitment of TSCs to an injury site *in vivo* has not yet been explored in detail, and studies to date have primarily been performed in cell culture. Structural differences to the tendon ECM with aging as discussed above may further alter the ability of TSCs to migrate to wounds *in vivo*.

Only a few studies have investigated the ability of aged or senescent TSCs to perform their duties with regards to matrix synthesis. One recent study revealed significant deficits in the ability to form three-dimensional tissue organoids, citing poor ability to produce and organize collagen matrix and reduced expression of matrix-related genes, including collagen I and key regulators of fibrillogenesis (Yan et al. 2020). In addition, organoids formed from aged TSCs also exhibited significant apoptosis and senescence. Expression of ECM and ECM-remodeling genes was found to be significantly reduced in other studies of aged mouse and human tendons, specifically reporting reduced collagen expression and reduced collagen production in aged TSCs (Klatte-Schulz et al. 2012; Han et al. 2017; Gehwolf et al. 2016). This could suggest that aged TSCs, and specifically senescent TSCs, may respond to injury via fibrotic mechanisms.

3.4.3.5 Altered Intercellular Communication and Mechanosensing

Tissues are able to grow and function normally due to the ability of cells to communicate with each other, constantly transferring information locally to nearby cells through direct cell-cell junctions or through the interstitial matrix via secretion of soluble factors (López-Otín et al. 2013; Rebelo-Marques et al. 2018). Aging can alter the ability of cells to perform this function and in the case of stem cells, impact cell fate and function. Signaling in tenocytes during development, homeostasis and injury has been extensively studied as it is critical to transduction of mechanical signals in order to facilitate tissue adaptation (Wall et al. 2016; Wall and Banes 2005). Dysregulated cell-cell communication was reported in aged TSCs recently (Popov et al. 2017), but interestingly this has not been explored in aging tenocytes yet. This avenue of investigation may be critical to understanding the dysfunction of matrix maintenance that occurs with age and we strongly encourage more research in this area.

3.5 Novel Systems and Tools to Study ECM Maintenance and Regulation

At the heart of the research discussed above is the dynamic addition and removal of material from critical structures within the tissue. The net flux of molecular components to developing and extant structures is positive during matrix assembly/growth, zero during maintenance and negative during degradation. For collagenous tissue assembly, maintenance and dysregulation it is critical to track (1) the production/export of new ECM molecules, (2) the degradation of existing ECM molecules, (3) the trafficking of ECM molecules from the cells to the matrix, and (4) the fate of the ECM molecules as they incorporate into matrix structures. This work heavily relies on novel tools and model systems used to track ECM molecules. Here we focus on those that can be used extracellularly, where they can help illuminate the dynamics of component exchange in the compartment that resides between the cells and the structural matrix.

3.5.1 In Vitro Model Systems

One major hurdle to studying ECM maintenance throughout life is the difficulty in measuring matrix production and breakdown in real-time without disruption of the intricate tissue structure. A number of simpler *in vitro* model systems have been designed to address this concern. Generally, *in vitro* culture allows for complete control and accurate measurement of applied mechanical and biological stimuli through the use of novel bioreactors (Wang et al. 2013a, b; Dyment et al. 2020; Janvier et al. 2020; Tohidnezhad et al. 2020; Chen et al. 2016; Butler et al. 2009), allowing for simple and straightforward experiments. Recent developments in tissue engineering strategies have allowed researchers to produce three-dimensional tissue engineered constructs (TECs), bioartificial tendons (BATs), and ligament equivalents (LEs) (Chen et al. 2016;

Deng et al. 2009; Butler et al. 2008; Garvin et al. 2003; Huang et al. 1993). Typical cell sources for engineered neo-tendons include mesenchymal stem cells, fibroblasts, embryonic tendon cells, and tendon progenitor or stem cells (TSCs), which are harvested and expanded using traditional culture methods. Cells are then supplied with appropriate growth factors and mechanical cues to stimulate production of tendon-like matrix. Mechanical cues include the use of custom bioreactors to stimulate tenogenic differentiation through static and cyclic tensile loading as well as the use of spatial or organizational cues, such as high aspect ratio channels and aligned substrate morphology in order to stimulate cells to form aligned tendon-like collagenous tissue.

Through this work, researchers have established that mechanical stimulation is critical for formation of appropriate collagen fibril morphology *in vitro (*Kalson et al. 2011*;* Mubyana and Corr 2018*;* Schiele et al. 2013*;* Kapacee et al. 2008*)*. The arrangement of geometric constraints (posts, channels, etc.) and the topographical surface in these systems can dictate both matrix alignment and cell phenotype, opening the door for studying links between substrate-specific mechanotransduction and matrix assembly (Schiele et al. 2013; Nirmalanandhan et al. 2007; Bayer et al. 2010). Furthermore, these systems have been critical in identifying which cell types can be induced to a tenogenic lineage and the necessary conditions to do so (Chen et al. 2016; Rajpar and Barrett 2019; Angelidis et al. 2010; Harris et al. 2004). These studies have paved the path for *in vivo* studies using larger and more complex tissue engineered constructs for tendon repair and also aided in the establishment of metrics to define repair capacity for tendon-derived cell populations, all while revealing the sophistication and complexity of tendon and ligament cell biology. However, these neo-tendons and more sophisticated TECs have not been able to faithfully recapitulate the mature tendon matrix, lacking hierarchical fibrillar structure and mechanical integrity, and thus can only be used for studying the initial stages of ECM production and not adult maintenance. Furthermore, studies to date have only focused on the collagenous matrix and have not investigated the interfibrillar and interfascicular matrix development. Finally, the study of age-related dysfunction would be difficult in systems requiring cell expansion due replicative senescence-prone aged cell populations.

Though the technique has been used since the late 1980s (Dyment et al. 2020; Wunderli et al. 2020), explant culture models have gained popularity again recently to study matrix turnover *in vitro* without major disruption of the hierarchical ECM structure. Explants can be harvested either as whole tendon with adjacent muscle and bone intact (murine rotator cuff (Connizzo and Grodzinsky 2018b)), intact tendon midsubstance (canine (Hannafin et al. 1995; Ikeda et al. 2010), rabbit (Abrahamsson et al. 1991), equine (Murphy and Nixon 1997), avian (Flick et al. 2006), and murine flexor tendon (Connizzo et al. 2019)), functional tendon sub-units (rat tail tendon fascicle (Lavagnino et al. 2016; Wunderli et al. 2017; Leigh et al. 2008; Screen et al. 2005b)), or cut pieces of tendon (human (Wong et al. 2009; Costa-Almeida et al. 2018) and bovine tendon explants (Koob and Vogel 1987; Samiric et al. 2006)). Historically, these explant models have been used primarily to understand the role of mechanical stimulus in preventing degeneration of tissue ECM either by studying stress deprivation or applying mechanical stress or strain to explants via custom-built bioreactors (Koob and Vogel 1987; Lavagnino et al. 2003; Connizzo et al. 2019; Hannafin et al. 1995; Flick et al. 2006; Gardner et al. 2012). They have also been used more recently to study inflammation, disease, and injury through the use of medium additives and other chemicals to simulate various biological environments (Connizzo and Grodzinsky 2018b, 2020; Abrahamsson et al. 1991; Wong et al. 2009; Fessel et al. 2014). Combining these model systems with the use of traditional labeling pulse-chase experiments allow for the measurement of matrix (proteoglycan, collagen) synthesis (Connizzo and Grodzinsky 2018b; Koob et al. 1992; Robbins et al. 1997). Recent studies have identified sex- and age-related differences in matrix synthesis and overall tissue metabolism despite no initial

differences at baseline (Connizzo et al. 2019), highlighting the power of these model systems in studying cell-mediated ECM remodeling in real-time.

A major benefit of explant culture models is the preservation of the intact hierarchical fibrillar matrix and the internal cell population, allowing for study of natural cell-matrix interactions and more minor changes to the ECM. Furthermore, it is possible that explants could be a viable model system to study the interfibrillar matrix, as this tissue is also kept intact during harvest. Explant tissues can be harvested from transgenic and aged animals, allowing us to pinpoint the roles of certain regulatory proteins in ECM homeostasis directly as well as to capitalize on the use of previously established injury and disease models. However, explants are inherently separated from other cell types and tissues that may be relevant for ECM maintenance, such as systemic innervation, lymphatics, and vasculature. Furthermore, tissue harvest induces injury and appropriate culture conditions for tissue maintenance is still an ongoing avenue of investigation by multiple laboratories (Abrahamsson et al. 1991; Wunderli et al. 2017; van Vijven et al. 2020; Vogel and Hernandez 1992). Nevertheless, with the increase in novel tools for measuring real-time ECM regulation, there is untapped potential for explant culture systems in studying ECM turnover.

3.5.2 In Vivo Model Systems

In vivo animal models, and in particular transgenic rodent models, have long been used in tendon research to study tendon matrix development, maturation, and aging (Delgado Caceres et al. 2018; Hast et al. 2014; Carpenter et al. 1999; Robinson et al. 2017). However, traditional transgenic animal models are limited by an inability to separate temporal regulation and compensatory effects due to the involvement of many regulatory proteins during tendon development (Connizzo et al. 2013a; Theodossiou and Schiele 2019). However, precise control of expression via the establishment of novel inducible mouse lines have since allowed for the study of temporal expression patterns during healing and growth (Gumucio et al. 2020; Disser et al. 2019; Ackerman et al. 2017c), as well as the ability to label cell populations for lineage tracing and local expression pattern studies (Yoshida et al. 2016; Soeda et al. 2010; Dyment et al. 2014, 2015). Using tamoxifen-inducible scleraxis-cre mouse models, researchers recently established that decorin and biglycan contribute critically to normal tendon homeostasis and aging, despite their low expression relative to developmental time points (Robinson et al. 2017; Leiphart et al. 2020). However, one study demonstrated deleterious effects of tamoxifen injection on tendon homeostasis and healing (Best et al. 2020) alluding to pro-fibrotic mechanisms, and another demonstrated altered rotator cuff healing (Cho et al. 2015), warranting further exploration. Using a doxycycline-induced green fluorescent protein (GFP) reporter model, researchers were able to pinpoint the transition from development to homeostasis, and correlate this to tissue growth (Grinstein et al. 2019).

In addition to these inducible models, the generation of tendon-specific knockout mice and cell lines via targeting of scleraxis-lineage cells has revolutionized the study of tendon development, homeostasis, and aging (Gumucio et al. 2020; Yoshida et al. 2016; Pryce et al. 2007; Schweitzer et al. 2001; Killian and Thomopoulos 2016). First, the study of key ECM regulatory proteins that were previously unexplored due to embryonic or perinatal lethality is now possible. Through these efforts an essential role for collagen XI in tendon development was discovered where the lack of collagen XI resulted in altered fibrillar structure and organization, as well as reduced tissue function (Sun et al. 2020). In addition, these models have established a critical role for MMP-14 during development in the formation of collagen fibrils, in sharp contrast to more well-known function in facilitating matrix breakdown (Taylor et al. 2015). Furthermore, recent work has identified the critical role of collagen V in the regulation of regionally-dependent (Connizzo et al. 2016b, c) and site-specific tendon structure and function (Sun et al. 2015b; Connizzo et al. 2015). Along with novel induc-

ible models, these technologies are primed to study the role of matrix regulators in tendon homeostasis without disruption from developmental processes or systemic changes associated with genetic knockdown.

Despite these major advances, there are still a number of hurdles in studying tendon matrix homeostasis and regulation. One difficult area of study is the dysfunction of ECM regulation during aging. Mice considered for the study of aging should ideally be between 18 and 24 months of age, after most biomarkers of aging are present and before survivorship drops significantly (Flurkey et al. 2007). Maintaining aging rodent colonies is both expensive and time-consuming, especially when considering novel transgenic lines for which breeding must be performed in house. One solution is to consider mouse models of accelerated aging, such as models of progeroid syndromes, models of mitochondrial mutations, senescence-prone mice or models of 'inflammageing' (Folgueras 2018; Kõks et al. 2016; Butterfield and Poon 2005). We are not yet aware of any studies investigating tendon aging with these models nor is there much evidence of tendon disease, and therefore this presents an interesting future avenue of exploration.

3.5.3 Tools for Labelling Collagen Turnover

The advent of electron microscopy (EM), its application to living systems and the recent extension of its capacity to produce highly-detailed 3-D serial reconstructions of tendon nanoscale structure has advanced our understanding of tendon morphology and development tremendously (Fig. 3.5a, b) (Birk et al. 1990b; Starborg et al. 2013; Trelstad et al. 1982). EM has sufficient resolution to observe the details of cell/matrix interaction with nearly molecular resolution. However, EM requires dehydration and fixation of tissue, it thus cannot address the critical question of directionality or magnitude of the flux of molecules, leaving matrix assembly and degradation dynamics an indirect and speculative endeavor. While it is possible to image "single" collagen fibrils and collagen matrix remodeling with label-free methods such as second harmonic generation (SHG, Fig. 3.5c) (Campagnola et al. 2002; Cox et al. 2003; Theodossiou et al. 2006), differential interference contrast (DIC) (Petroll and Ma 2003; Bhole et al. 2009) and confocal reflection (Brightman et al. 2000; Kim et al. 2006), these methods are also limited by a number of constraints: SHG reportedly has nanoscale resolution [recently claimed at 30 nm (Bancelin et al. 2014)] but only captures fibrils with non-centrosymmetric organization because it relies on a lack of inversion symmetry (Campagnola et al. 2002). DIC cannot resolve fibrils in dense tissue and is subject to orientation angle contrast dependency (Siadat et al. 2021a) and confocal reflection microscopy has resolution limitations and density/contrast difficulties as well. In addition, all of them are unable to track the fate of single molecules during their transit to and from the matrix.

The ultimate goal of labelling is to determine the spatial and temporal fates of target molecules from their translation to the site of action to removal from service, all in real time in a living animal. It would be even better if their exact locations and orientation with structures could be determined as well (Alzola et al. 2021). Fortunately, the labelling and tracking of matrix molecules has been proceeding apace for years secondary to advances in labelling techniques and microscopy methods. As far as we can tell, no combination of molecular probe and imaging method has met this lofty standard to study tendon extracellular matrix. However, there are a number of probe/microscopy combinations that can reasonably be used to ask particular, circumscribed questions with excellent results.

3.5.3.1 Collagen-Binding Protein Labels

Collagen labels based on a bacterial adhesion protein with specificity for collagen (CNA35) and on an integrin (GST-α_1I) were recently demonstrated in Krahn et al. (2006) The labels were shown to be more specific than dichlorotriazinyl aminofluorescein (DTAF) which has been the standard for tracking collagen formation. CNA5

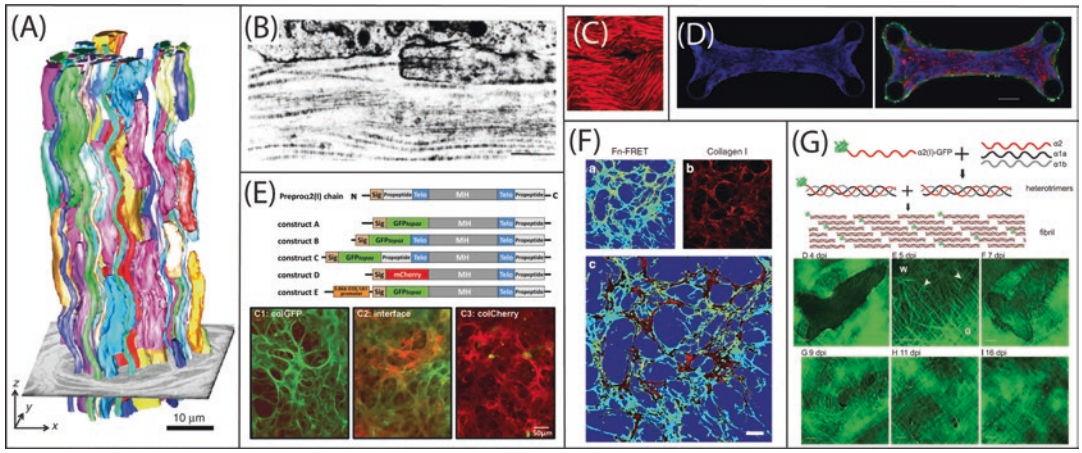

Fig. 3.5 Tools for labelling collagen synthesis, remodeling, and incorporation. (**A**) 3View® analysis of resin embedded sample of a newborn mouse tendon. Colors represent different cells/bundles of fibrils. From Starborg et al. (2013) with permission. (**B**) Conventional transmission electron micrograph of collagen formation in a developing chick tendon showing the details of the cell-fibril interface. From Trelstad et al. (1982) with permission. Scale bar is 300 nm. (**C**) Label free, confocal SHG images of collagen in frozen rat foot flexor tendon in transmission mode; 880 nm pumping frequency. From Theodossiou et al. (2006) with permission. (**D**) Labelling of engineered collagen-rich cardiac tissue. Collagen is stained with reversible collagen binding dye produced in bacteria: CNA35-m Turquoise2. Cells: green; mitochondria: red. Modified from Aper et al. (2014) with permission. Scale bar is 100μm. (**E**) Endogenous label incorporation The GFPtopaz and mCherry labels indicate that individual collagen molecules are incorporated into the same network of forming fibers. From Lu et al. (2018) with permission. (**F**) Fret labelled Fibronectin (Fn-FRET) and type 1 Collagen mechanochemical interaction probed from Kubow et al. (2015) with permission. Collagen was shown to colocalize principally with unloaded FN (yellow). Scale bar is 20μm. (**G**, top) GFP labelled collagen I zebrafish line generation. The N-terminal region of the collagen I α2 chains were selected for placement of the label. GFP-tagged alpha chain trimerises with unlabeled "**a**" and "**b**" chains. A mix of heterotrimers (labeled and unlabeled) are capable of forming fibrils with the label residing in the intratrimer gaps. (**G**, bottom) Progression of the closing of incision wound in the flank skin of transgenic zebrafish. Wound gape due to tension release shown at 4 dpi is closed with loose network of deposited collagen fibrils showing poor organization at 5 dpi. The collagen network is repaired over the next 11 days and reorganized into an orthogonal pattern by 16 dpi. Scale bars are 15μm. (Modified from Morris et al. (2018) with permission)

had better affinity for collagen than GST-α_1I and did not show substantial cross-reactivity with NCPs in the matrix. The binding of the probe is reversible which makes it "unlikely" to affect matrix production and permits time course investigations of matrix development. However, the probe is not specific for type I and also binds collagen III and IV. An interesting application for the probe was one in which the probe was bound to collagen and made "activatable" via MMP-2 proteolysis (Xia et al. 2011). More recently, the same group added six genetically encoded collagen probes produced in bacteria that fuse CNA35 to fluorescent proteins across the visible spectrum in an engineered, collagen rich tissue (Fig. 3.5d) (Aper et al. 2014). There have been multiple collagen binding proteins discovered which can be used to label collagen (Chilakamarthi et al. 2014), which would all operate in a manner similar to CNA35. While the primary utility of these probes is the real-time, multi-color imaging of live tissue, *in situ*, the multiple color probes could make it possible to perform sequential collagen deposition tracking experiments provided the reversibility of previously bound probe does not permit exchange with newly added probes. However, the size of the probe could inhibit proper assembly of matrix given that the molecular weight is the combination of the CNA35 (35 kDa) and the fluorophore (e.g. 93 kDa for tdTomato). Furthermore, there is a troubling lack of investigations, demonstrating the effect of collagen binding proteins on collagen assembly kinetics.

3.5.3.2 Bio-orthogonal Labels

Advances in bio-orthogonal chemistry have led to the development of a series of functionalized metabolites that act as chemical reporters (Grammel and Hang 2013; Dieterich et al. 2006). These can be viewed as analogous to the early radio tracer experiments with the exception that they are non-toxic, easily incorporated with little regulation, have low off target effects and can be readily illuminated fluorescently with high resolution, *in vitro* and *in vivo*. The process involves separating the incorporation of the reporter from its detection. This prevents the addition of bulky fluorophores until a readout is desired, which also presents opportunities for pulse chase experiments. Proteome tagging using non-canonical amino acids with reactive handles has the potential to revolutionize live cell imaging and tracking of molecular moieties. Non-canonical amino acids are incorporated into the target molecule using the cell's own machinery. Amgarten et al. labelled collagen with azido-proline (N_3-Pro) in fetal ovine osteoblast culture via supplementation of the growth medium with cis-4-azido-L-proline (Amgarten et al. 2015). The incorporation of the N_3-Pro minimally affects collagen formation as expected, and provides a substrate for dibenzooctyne (DIBO) fluorescent probe. While they show that the N_3-Pro did not affect cell viability, the DIBO reacted with some intracellular components including actin increasing background fluorescence which required additional treatment to reduce. Nonetheless, bio-orthogonal collagen labelling has enormous potential as a live cell and *in vivo* imaging technique. In 2014, Mirigian et al. performed bio-orthogonal pulse chase experiments in dermal human fibroblasts with and without a type I collagen chain mutation (Mirigian et al. 2014). They incorporated the non-canonical amino acid azidohomoalanine (Aha), a methionine (Met) analog, into cell secreted collagen by supplementing Met and Cys-free DMEM with Aha. They reported quiet incorporation of the Aha into the collagen with no discernible effect on post-translational modification, stability or structure of the triple helix. The utility of the tracing was demonstrated by successful measurement of pro-collagen folding kinetics in a normal and osteogenesis imperfecta patient's cells, which is a highly challenging pulse-chase experiment due to the short pulse window.

3.5.3.3 ECM Proteins Conjugated to Labels

Rather than add proteins or peptides that target and bind to ECM components already in the tissue, it is sometimes possible to add labelled ECM proteins themselves to the system as participating tracking molecules. The theory behind this approach is that ECM proteins will behave as they would whether they are secreted by the cell or added to the system. Collagen has a long history of being directly labelled and added exogenously to living systems where it has shown an ability to "home" to its proper morphological position. Stopak et al. injected covalently labelled (FITC) collagen type I into chick limb buds to track its incorporation into tissue rudiments including tendon (Stopak et al. 1985).

In an excellent demonstration of the utility of conjugated ECM protein labels, Sivakumar et al. added fibronectin (FN) conjugated to AlexaFluor 488 or 555 (Sivakumar 2006). These FN labels were dynamically tracked throughout the construction of matrix by osteoblast cells in a culture system showing a remarkable view of matrix assembly dynamics (Kadler et al. 2008). In an extension of this concept, exogenous FN labelling can be adapted in conjunction with Förster resonance energy transfer (FRET) to produce mechano-sensitive imaging (Kubow et al. 2015). In a seminal report, Kubow et al. added FRET labels to plasma FN such that mechanical unfolding of the molecule displaced the FRET labels and produced a detectable signal in a live culture system (Fig. 3.5f). The co-localization of the fibronectin FRET signal with collagen (immunolabelled) permitted the observation of collagen and FN interaction principally when the FN was relaxed and not under load. The collagen-FN mechanochemical reciprocal relationship was also recently probed in a cell-free system whereby collagen fibril nucleation was catalyzed by FN under conditions of extensional strain (Paten et al. 2019). Because labels can interfere with

functionality of the protein, efforts have been made to reduce the size and degree of labelling of the probe (Siadat et al. 2021b). An interesting alternative approach was recently described by Doyle which attempts to preserve the intermolecular lysines for association in fibrils rather than labelling sites (Doyle 2018). To do this, Doyle labels the collagen (atto-488 NHS-ester dye) as a formed gel, then reverts the gel back to the molecular state and dilutes the labelled monomers with unlabeled collagen (~2:98%). Reformation of the mixed collagen produces a bright collagen network suitable for cell culture.

3.5.3.4 Endogenous Labels

Some of the most impressive work has been done with endogenous labels in live cultures and in living animals. While *in vitro* systems have substantial and well-known limitations relative to *in vivo* systems, there are a number of advantages which permit excellent observational fidelity. One of the more striking examples of *in vitro* imaging of labelled collagen assembly dynamics was performed using the osteoblast-like cell line MLO-A5 (Lu et al. 2018). The cells were transfected with GFPtpz and mCherry-collagen expression plasmids with careful attention paid to the placement of the label (Fig. 3.5e). The dual collagen labels permitted the dynamic observation of the interface that developed between differentially-labelled cell systems. Co-cultures of two different colored collagen expressing cells, produced a collagenous ECM that fused both colors, indicating a mixing of collagen molecules from each construct to form new fibrils (Fig. 3.5e). While labelling in cell culture is quite informative, it is always striking to see labelling performed well in a living system. In a recent and elegant paper, Morris et al. label type I collagen in a living zebrafish and dynamically track the progress of repair of a wound in skin (Morris et al. 2018). In their experiment, they drove expression of col1a2-GFP using a krtt1c19e promoter known to express in the basal epidermis which produces skin collagen type I in early development Tg(krt19:col1a2-GFP). The placement of the GFP label at the N-terminal region of the collagen molecule ostensibly minimizes the effect of the label on the assembly kinetics and morphology of collagen fibrils formed from them. This work stunningly demonstrates the progression of collagen disruption, organizational control and deposition during repair of a skin wound in the zebrafish (Fig. 3.5g).

3.5.3.5 Collagen Hybridizing Peptide

While labelling intact and functional collagen is informative, it is also quite important to develop labels which can identify collagen that is damaged. The principal role of collagen as a load bearing material makes understanding its failure mechanisms and subsequent repair critical to the development and timely application of clinical treatments for a broad range of injuries. Collagen molecular damage has been evaluated by a number of different methods including increased digestion susceptibility (Willett et al. 2007) and changes in denaturation endotherms (Willett et al. 2008). However, in 2012 Li et al. presented a paper on a caged collagen mimetic peptide (CMP) or collagen hybridizing peptide (CHP) which could be photo-triggered to fold into a triple helix capable of binding heat-denatured or MMP-digested collagen (Li et al. 2012). Zitnay et al. convincingly demonstrated that the CHP would bind preferentially to damaged collagen in 12% strain-overloaded rat tail tendon fascicles using transmission electron microscopy (TEM) and gold nanoparticle labelled CHP (Zitnay et al. 2017). The intensity of CHP staining of cyclically-loaded tendon increased with the frequency and number of the load cycles. More recently, the authors used this technique to measure the molecular damage to rat tail tendon fascicle collagen during cyclic fatigue loading (Zitnay et al. 2020), which has significant implications for our understanding of overuse injury.

3.6 Conclusions and Avenues for Future Work

We review here the large body of work investigating the formation, assembly, and maintenance of the tendon extracellular matrix. It is clear that a vast majority of this work has historically focused

on embryonic and postnatal development, and despite nearly a century of research, there are still knowledge gaps and debates among the experts regarding how collagen fibrils form and assemble into the intricate tendon hierarchical structure. The exact growth mechanisms in tendon are still currently unknown. We still do not understand how cells and matrix work together to establish initial continuity in the mechanical structure of developing animals. It remains unclear if mechanical force drives fibril assembly at the molecular level or if fibrils are synthesized first and then organized. While it has been established that traction forces applied by resident cells are necessary for fibril formation, the precise mechanism and location that cells use these forces to convert soluble collagen monomers into fibrils are still to be determined. Furthermore, the question of how fibrils lengthen in a growing tendon under load while preserving mechanical integrity remains unresolved. We are also still understanding how collagen molecules within a matrix that endures high mechanical forces and a large number of cycles have such a long half-life.

There are also still a number of open questions regarding the mechanisms of adult matrix turnover or adaptation. If we ultimately want to understand how chronic matrix degeneration occurs, as in the case of tendinopathy, we want to identify the initiators of matrix remodeling and what events would make this process go awry. One of the missing gaps in this field is a lack of understanding in the repair of sub-failure damage or microdamage and how these mechanisms are different from a massive injury response. In addition, it would be beneficial to know where and how microdamage is initiated and to develop methods to track this damage. Since mechanical function and tissue structure are highly dependent on functional needs, it's possible that the turnover of individual matrix proteins is also functionally specific. Protein turnover in functionally distinct tendons varies with protein type but relative turnover rates for individual proteins between tendon types remain to be determined. Moreover, it is possible that turnover at the junction of tendon with another dissimilar tissue, such as at the enthesis or the myotendinous junction, is more rapid than in the midsubstance. Answers to these questions would dramatically improve our understanding of adult tissue maintenance and potentially provide clues to chronic degeneration.

Age-related cellular mechanisms are likely to blame for the dysfunction of normal tissue homeostasis that could lead to chronic degeneration, but the mechanisms behind these deficits have not been fully established. It is uncertain whether there are changes in mechanosensing or mechanotransduction, preventing cells from sensing and converting appropriate mechanical signals to elicit remodeling, or whether the dysfunction is in the processes of matrix remodeling itself, limiting the synthesis, assembly or incorporation of new ECM. More work is needed to identify what these cellular changes are and how they influence the ability to maintain tissue architecture. In addition, while cellular changes have been studied extensively in tendon stem cells and particularly in relation to the injury response, fewer studies have investigated age-related changes in mature tenocytes which we expect to be responsible for local tissue repair in the absence of inflammatory cell recruitment. Finally, it is important to note that many of the 'hallmarks of aging' are extremely interconnected and most of them have not yet been directly investigated in tendon; therefore, there are likely aging mechanisms that influence matrix homeostasis that have yet to be uncovered.

There are also still major deficits in our basic knowledge of the tendon composition and structure, specifically in the non-collagenous matrix and the cell populations present. Much of the research presented has focused on regulation of the collagen structure, with considerably less attention placed on the regulation of the non-collagenous compartment, specifically the interfibrillar and interfascicular matrix as well as paratenon and epitenon. Studying the non-collagenous matrix is quite challenging due to low abundance and difficulty in precise extraction, as well as absence of *in vitro* systems focusing on it. In addition, *in vivo* models permitting

genetic modification (rodents) lack an interfascicular compartment posing another hurdle in the study of the non-collagenous matrix, whilst larger animal models (horse) have an interfascicular compartment but do not lend themselves to genetic modification and longitudinal studies due to time and cost constraints.

Furthermore, this chapter focuses on the regulation and dysregulation of the tendon ECM throughout life, all of which is cell-mediated. However, we still do not have a complete understanding of the specific cell populations that are present in whole tendon and their localization. Recent studies have focused on identifying and characterizing cell populations, highlighting the vast heterogeneity and complexity of the population within tendon compartments. With the advent of single-cell sequencing, investigation of cell heterogeneity within tissues has been made possible and its recent use in tendon research has unveiled several tendon cell subtypes that could be responsible for matrix remodeling (Paolillo et al. 2019; Harvey et al. 2019; Kendal et al. 2020; De Micheli et al. 2020; Yin et al. 2016). Therefore, there appear to be many different subpopulations of cells responsible for producing ECM but the role of the identified clusters in the development, maintenance, and aging of tendon still remains to be elucidated.

Many of these questions will still require years of research to answer, but the development of novel models and tools to study ECM remodeling provide substantial promise for future investigation. With the ability to label and track collagen, and hopefully someday non-collagenous proteins, mechanisms of matrix incorporation and linear growth that have evaded detection in previous years may now be uncovered. Increased knowledge of the processes controlling matrix growth and incorporation could provide guidance for tissue engineering approaches. Furthermore, if key regulators of matrix homeostasis during adulthood and into aging are identified, it may become possible to identify the tipping point between positive adaptation and degeneration leading to progressive tendinopathy. Not only will this allow us to understand the process of degeneration, it will also put research one step closer to developing therapeutics and/or preventative interventions for tendon injury and disease.

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