Published in final edited form as:
Curr Opin Biotechnol. 2013 October ; 24(5): . doi:10.1016/j.copbio.2013.07.003.

Mechanical factors in embryonic tendon development: Potential cues for stem cell tenogenesis

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Abstract

Tendons are connective tissues required for motion and are frequently injured. Poor healing and inadequate return to normal tissue structure and mechanical function make tendon a prime candidate for tissue engineering, however functional tendons have yet to be engineered. The physical environment, from substrate stiffness to dynamic mechanical loading, may regulate tenogenic stem cell differentiation. Tissue stiffness and loading parameters derived from embryonic development may enhance tenogenic stem cell differentiation and tendon tissue formation. We highlight current understanding of the mechanical environment experienced by embryonic tendons and how progenitor cells may sense and respond to physical inputs. We further discuss how mechanical factors have only recently been used to induce tenogenic fate in stem cells.

Introduction

Tendons serve a critical mechanical function by transferring muscle-generated forces to bone. Unfortunately, injuries lead to disorganized tissue structure and abnormal mechanical properties, despite surgical intervention. Alternatively, tissue engineering promises the replacement of injured tendon with new, normal tissue. Efforts have focused primarily on dynamic mechanical cues to induce and guide tenogenesis, but progress has been limited, presumably due to insufficient knowledge of tenogenic mechanical factors and their roles during normal tendon development. Notably, tissue elastic modulus and dynamic mechanical forces have been shown to regulate stem cell differentiation toward other lineages [1–4]. With the goal of enhancing strategies to mechanoregulate tendon regeneration, we are interested in how tendon cell fate decisions may be influenced by embryonic mechanical factors. This review examines current understanding of the mechanical microenvironment of tendon during embryonic development. Specifically, we focus on two primary mechanical factors, tissue modulus and dynamic loading, and efforts to identify these cues and their potential influences. Furthermore, we discuss mechanically driven mechanisms that may guide tenogenic fate decisions. Finally, we review recent studies that exploit mechanical loading to direct stem cell tenogenesis (differentiation

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toward the tendon lineage). Characterizing the mechanical cues involved in embryonic tendon development may provide parameters for scaffold design and bioreactor culture to mechanoactively guide tenogenic stem cell differentiation and tissue formation, thereby enhancing tendon tissue engineering strategies.

Embryonic tendon elastic modulus

Substrate stiffness has been shown to regulate stem cell differentiation toward the adipogenic, myogenic, neurogenic and osteogenic lineages [1–3,5], though this has been only minimally investigated for the tenogenic lineage [5]. It is not yet known how tendon progenitor cells might sense and respond to the mechanical properties of developing tendon tissue during embryonic development because until recently, data on mechanical properties of embryonic tendon have been limited and inconsistent. For instance, reported values for tensile elastic modulus of late-stage embryonic chick tendon have varied by nearly 100-fold [6,7] (Table 1), perhaps due to difficulties with mechanically testing small and delicate embryonic tissue. Bulk tensile properties of embryonic tendon are important for understanding tissue function, but represent properties at size scales and magnitudes significantly greater than cells. In contrast, cell length-scale mechanical properties may be more relevant for mechanoregulation of cell differentiation and function. Recently, we characterized nanoscale and microscale elastic moduli in developing embryonic chick tendon using atomic force microscopy [8], finding elastic modulus to be up to 49-fold lower than previous bulk level embryonic tendon measurements and up to 40,000-fold lower than that of adult tissue [9] (Table 1). While there is still much to understand about how cell length-scale mechanical properties influence tenogenesis, these studies provide a framework from which to begin to investigate such mechanisms.

Mechanical stimulation of tendon during embryonic development

In addition to mechanical cues from tissue stiffness, embryonic tendon cells (ETCs) likely experience mechanical loading via muscle contractions during development. Since muscular contractions begin relatively early in development, embryonic tendons may experience dynamic loading during important stages of differentiation and tissue formation. Embryonic motility begins early, after neuromuscular connections form [11] at developmental day 4 in chick embryos [12], embryonic day (E) 12.5–14 in mouse [11,13], and 7 weeks in human embryos [14]. Chick embryos are active 20% of the time at developmental day 6 and nearly 80% starting from day 11, based on at least one movement every 10 seconds [12]. *In ovo* electromyography (EMG) recordings of chick embryonic gastrocnemius muscle showed motor unit activation every 2 seconds at developmental day 7 [15] and every 0.2 seconds by day 19 [16]. EMG activity is not equivalent to force or strain, but suggests that tendons experience muscle-derived forces during development at rates of 0.5–5 Hz. Multiple muscular loads may produce complex loading regimes [17], but how patterns of muscular activity translate to mechanical forces experienced by developing tendon cells requires further investigation.

Effects of muscle activity on tendon development

Reduced or altered skeletal muscle contraction during embryonic development produces significant skeletal abnormalities [18–25]. Chick embryo paralysis by D-tubocurarine from developmental days 10–18 inhibited formation of the tendon synovial sheath and fibrocartilaginous regions of embryonic digital flexor tendons [25]. Other studies using neuromuscular blocker decamethonium bromide to induce paralysis during embryonic chick development demonstrated tendon degeneration and a reduction in tendon size [22,23], and decreases in tenascin-C gene and protein expression [24]. In adult mice, botulinum toxin A-induced muscle paralysis decreased the number of scleraxis-positive (basic helix-loop-helix

transcription factor specific for tendon [26]) tendon cells by nearly 80%, along with decreases in collagen fibril density and stiffness [27]. In the same study, isolated primary tendon cells cultured under static conditions reduced scleraxis expression over time, whereas mechanical stimulation via fluid shear stress rescued scleraxis expression, which may have been mediated by the transforming growth factor (TGF-β) type I receptor and Smad 2/3 [27]. Chick ETCs express the TGF-β type I receptor [28], however the role of this receptor in mechanoregulation of embryonic tendon is unknown. Taken together, muscle contractions seem important for normal embryonic tendon development and homeostasis, however a potential non-mechanical confounding factor may be altered biochemical signaling from muscle tissues with paralysis. Other approaches to study effects of muscle loading *in vivo* have assessed embryonic tendon development in muscleless limbs. In the absence of muscle, initial tenogenic induction and progenitor cell distribution are unaffected in chick, but further tendon development is unable to proceed in the absence of muscle [29,30]. It is unknown if this dependency on muscle is a function of muscle-derived mechanical stimulation, secreted soluble (e.g., growth) factors, or both. It may be a combinatorial effect, as scleraxis expression is rescued with application of fibroblast growth factor (FGF)-4 in the absence of muscle [31]. Future studies are needed to delineate individual muscle-derived mechanical and biochemical effects on tendon development.

Quasi-static tension and compressive loading

Developing embryonic tendons may also experience quasi-static tension associated with limb lengthening. For example, the embryonic chick toe increases in length from HH 36–43 at a rate of 1.9 mm/day [32]. Recently, slow mechanical stretch was applied to chick ETCs seeded in fibrin gels *in vitro*, leading to increased collagen type I gene expression, collagen fibril size, volume fraction, ultimate tensile stress, elastic modulus and cell nuclei length [7].

Typically associated with tension, tendons also experience compressive and shear strains when wrapping around joints and contain fibrocartilaginous tissue in these regions [33,34]. Compressive loading of adult bovine flexor tendons upregulated synthesis of large proteoglycans *in vitro* [35]. Similarly, cyclically compressed embryonic bovine tendons upregulated aggrecan and biglycan gene expression *in vitro* [36]. TGF-β1 treatment enhanced expression of proteoglycans and TGF-β1, indicating positive feedback between loading and TGF-β1 [36]. These studies suggest specific mechanical stimuli can direct tendon progenitor cells toward distinct phenotypes within tendon tissue.

Mechanosensing mechanisms in tendon cells

Cells have force sensors to receive and transmit mechanical stimuli as biological signals. Though minimally characterized in ETCs, studies with adult tendon cells provide insight into potential mechanisms of mechanotransduction. Mechanosensing and mechanotransducing mechanisms in ETCs may occur through direct cell-to-cell connections (e.g., gap junctions and cadherins) or cell-to-extracellular matrix (ECM) adhesion molecules (e.g., integrins) (Figure 1). Downstream, cell cytoskeletal components may also respond to the mechanical environment.

Adult tendon cells *in vivo* maintain a network of gap junction proteins, connexins (Cx) 32 and 43 [37,38]. Gap junctions connect the cytoplasm of adjacent cells, allowing direct cellular communication and the transport of small molecules. Cx 32 and 43 are also present in cells throughout the limb bud and tendon during embryonic development [38–40]. Cx 32 appears to link tendon cells longitudinally, while Cx 43 links all adjacent cells, laterally and longitudinally [37]. Blocking gap junctions with octanol treatment in chicken tendon cells inhibited stimulation of DNA and collagen synthesis by cyclic loading, suggesting gap junctions play a role in mechanotransduction [41]. A more recent study demonstrated Cx 32

and 43 in chicken tendon cells respond differentially to mechanical stimuli. Antisense downregulation of Cx 32 reduced the stimulatory effect of mechanical loading on collagen synthesis, while antisense downregulation of Cx 43 enhanced collagen synthesis [42], suggesting they work in opposition. Cx 32 only links tendon cells along the tendon long axis [37], therefore the authors suggested that load in this direction may stimulate collagen synthesis, though Cx 43 co-activation may mitigate collagen production in response to mechanical signals [42]. Taken together, gap junctions and the molecules they transport may play mechanoregulatory roles in tendon development to produce a coordinated and directed cellular response to mechanical stimuli.

Cadherins are cell-to-cell adhesion proteins, which may also function as force sensors and mechanotransducers [43]. Cadherin-11, in particular, is highly expressed in embryonic tendon [44]. Downregulation of cadherin-11 with siRNA in chick embryonic tendon at developmental day 13 resulted in a loss of contact between adjacent ETCs and disrupted collagen fibril organization [44]. These results demonstrated that cadherin-11 maintains cell-to-cell contact and plays a role in collagen fibril organization during embryonic tendon development.

In addition to cell-to-cell connections, ETCs express ECM-specific integrins, which may play a role in mechanosensing and subsequent mechanotransduction pathways [45]. During early stages (developmental day 4) of embryonic chick development, integrin $\alpha 5\beta 1$ was found throughout the limb mesenchyme and at later stages localized in the developing connective tissues [46]. Mesenchymal cells express integrin $\alpha 11\beta 1$ during embryonic development [47,48], which, interestingly, was seen to be expressed in a similar pattern as scleraxis [48], suggesting a role in tenogenesis. As integrins have been shown to act as mechanosensors in other cell types and tissue systems, future studies should focus on whether and how tendon progenitor cells interrogate their physical environment via these transmembrane proteins.

The actin cytoskeleton provides structural integrity to tendon cells [49] and may participate in mechanotransductive signaling pathways [50]. *In vivo,* actin fibers in adult tendon cells follow collagen crimp patterns along the longitudinal axis (stretch direction) of the tendon [51]. When chicken tendon cells were mechanically strained in culture, tropomyosin protein content increased, suggesting enhanced actin fiber assembly [51]. Recent work with isolated primary chick ETCs demonstrated these cells contain actin fiber motors, nonmuscle myosin II (NMMII) heavy chain proteins, IIA and IIB [10]. Prior studies have shown that NMMII regulates cellular tension and mechanotransduction, affecting stem cell fate decisions [52,53]. Interestingly, transcripts for nonmuscle myosin heavy chain proteins IIA and IIB were higher in ETCs on tissue culture plastic than in those in soft 3D fibrin gels [10]. However, collagen type I gene expression was elevated in soft fibrin gels, compared to hard tissue culture plastic. These changes in gene expression may reflect a dependence on substrate stiffness, but there were confounding factors such as 2D vs 3D culture or altered integrin binding due to substrate material. In the same study, both NMMII and actin inhibition abolished expected increases in fibrin gel elastic modulus and ultimate tensile stress *in vitro* [10], suggesting that actin and NMMII interactions in ETCs are required for the development of engineered tissue mechanical properties. More work is needed to identify specific mechanotranductive signaling pathways that regulate tenogenesis during development.

Mechanoregulation of stem cell tenogenesis

In vitro studies have examined how dynamic tensile loading influences tenogenic gene expression in stem cells (Figure 2). Cyclic strain applied to C3H10T1/2 murine

mesenchymal stem cells in collagen gels upregulated scleraxis expression levels over static conditions [54]. In a number of studies, cyclically strained human mesenchymal stem cells (MSCs) maintained or upregulated tenogenic genes (scleraxis, collagen types I and III, tenascin-C) and increased matrix production [55–57]. Dynamic strain also increased focal adhesion kinase (FAK) phosphorylation in MSCs [55,56]. When FAK phosphorylation was inhibited, expression levels of collagen types I and III, tenascin-C, and scleraxis were reduced [55,56]. Similarly, actin disassembly and RhoA/ROCK signaling inhibition abolished the tenogenic response to dynamic strain [56]. Taken together, stretch-induced tenogenic gene expression in stem cells may be mediated by FAK, the actin cytoskeleton and RhoA/Rock signaling pathways. Further work is needed to characterize these and other potential mechanisms of mechanotransduction in tenogenically differentiating cells.

MSCs isolated from rat bone marrow were examined in collagen constructs that experienced regions of either tension or compression [58]. MSCs experiencing tension became aligned and elongated, and upregulated scleraxis and collagen type I gene expression, relative to compressed regions. TGF-β3 supplementation abolished scleraxis expression in either tensile or compressive regions, and increased aggrecan expression [58]. These results demonstrate that while tensile loading is tenogenic, compressive loading enhances a fibrocartilage-like phenotype in MSCs (Figure 2), and TGF-βs may play a role in this process. Currently it is unclear through what mechanisms tendon progenitor cells sense and respond differentially to tensile and compressive loads.

Recent work has also demonstrated that substrate stiffness may influence tenogenic stem cell differentiation (Figure 2). Human bone marrow stromal cells had increased scleraxis, tenomodulin, tenascin-C and collagen III gene expression on collagen-coated substrates with an elastic modulus of 40 kPa, relative to 20 kPa and 80 kPa [5], suggesting that substrate stiffness may mediate tenogenesis. Elastic modulus may be an important cue for tenogenesis by adult stem cells and deserves further investigation.

Conclusions and future directions

The complex mechanical environment that embryonic tendon cells experience encompasses physical factors from tissue stiffness to dynamic loading. By studying the mechanical microenvironment and mechanically regulated mechanisms involved in embryonic tendon development we may identify physical requirements for tenogenic stem cell differentiation. Toward that end, additional studies are needed to identify mechanical stimuli that elicit robust tenogenic signaling in differentiating ETCs. The chick has been the dominant embryonic animal model with which to study the role of mechanical influences in tendon development, presumably for its accessibility and relatively short developmental program (~21 days). However, while mechanisms of chick tendon formation overlap significantly with that of mouse [26], discovery and validation of mechanical cues with mammalian animal models and engineered systems will be necessary to advance human stem cell-based tendon regeneration strategies. This review focused on embryonic development, the period when stem and progenitor cell lineage commitment and differentiation occur, though understanding early postnatal events will be important in directing the continued development and maturation of engineered tendon as a functional tissue [59]. Characterizing mechanically regulated pathways during embryonic development may provide cues to guide engineered tissue formation and regeneration with stem cells and improve tissue engineering outcomes.

Acknowledgments

The authors would like to thank Jeffrey Brown, Ph.D. of Tufts University for critically reading this review. We would also like to thank our funding support by Research Grant 5-FY11-153 from the March of Dimes Foundation (to C.K.K.) and Award Number R03AR061036 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health (to C.K.K.).

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Highlights

- Mechanical influences on tendon cells during embryonic development are reviewed.
- Potential mechanisms of mechanotransduction in embryonic tendon cells are discussed.
- Mechanoregulation strategies to induce tenogenesis of stem cells are examined.

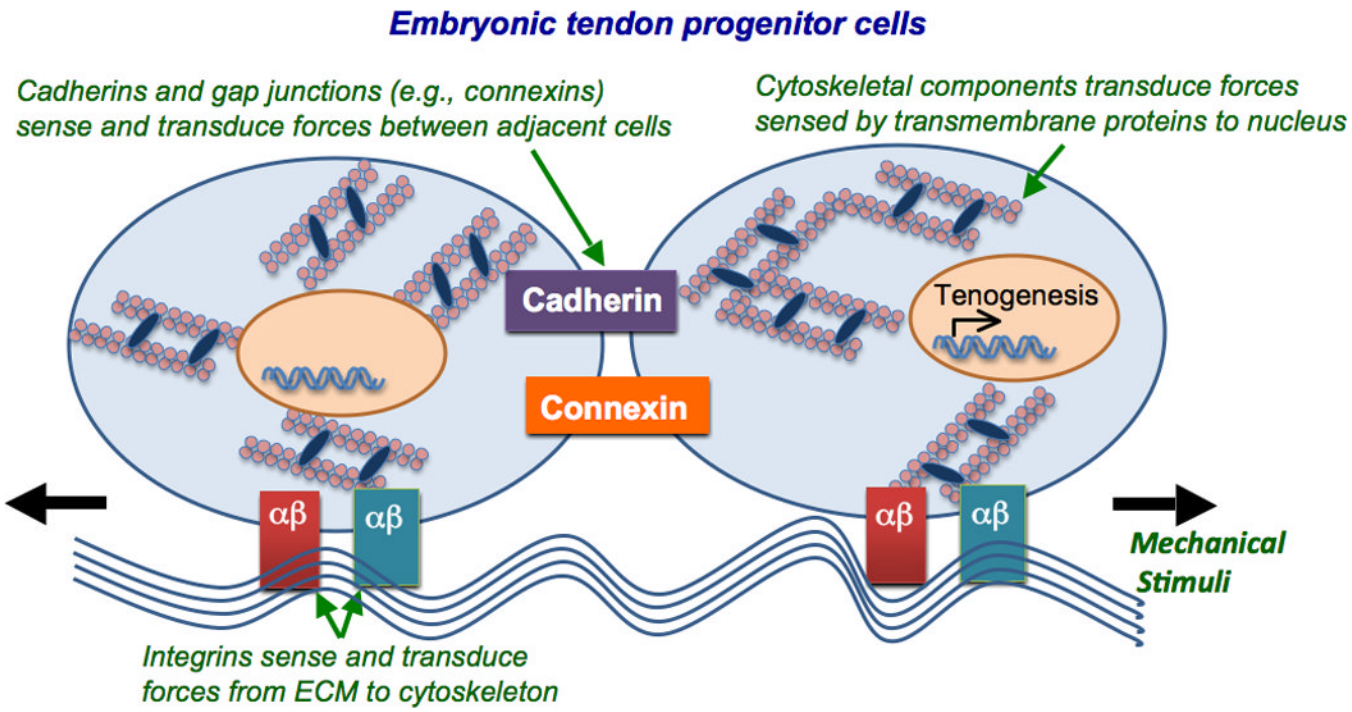

Figure 1. Mechanotransductive components of embryonic tendon cells

ETCs may sense and transduce mechanical signals between cells via direct cell-to-cell contacts such as cadherin (purple) and connexin (orange), and from the surrounding ECM via integrins (red and green). Downstream, cytoskeletal components that link to these transmembrane proteins may transduce forces to the nucleus to regulate gene and protein expression.

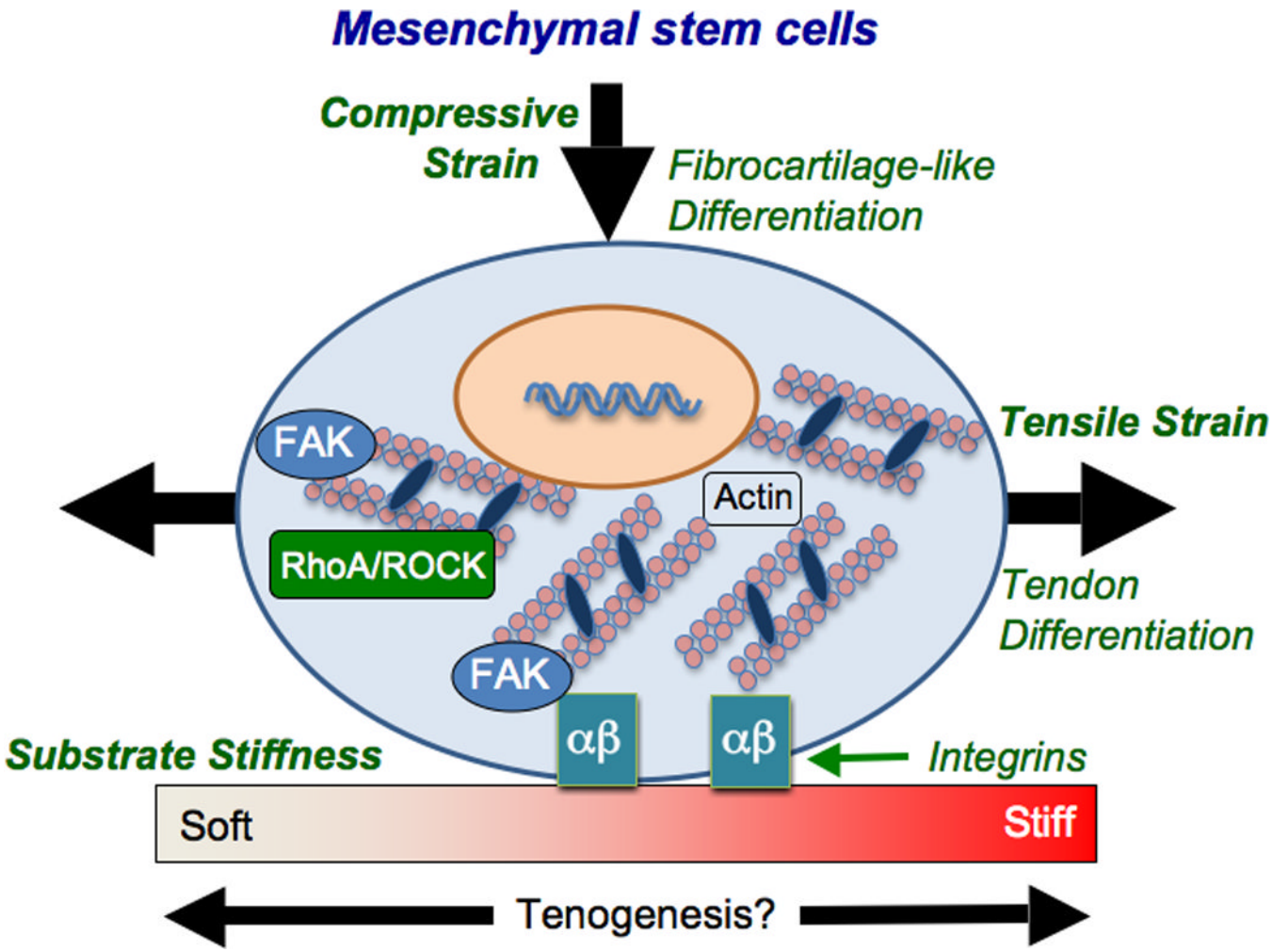

Figure 2. Mechanical factors may influence tenogenic differentiation of mesenchymal stem cells

The tenogenic effect of dynamic tensile strain may be mediated through FAK, RhoA/ROCK and the actin cytoskeleton in MSCs. While tension is tenogenic, compressive loading may induce a fibrocartilage-like phenotype. Substrate stiffness may provide an additional mechanical signal to influence stem cell tenogenesis.

Table 1

Elastic modulus values for embryonic and adult chicken tendon.

Developmental stage (HH)	Approximate embryonic days	Measurement method	Average elastic modulus range	Reference
HH 40–43	Day 14–18	Bulk tensile test	0.21–1.02 MPa	McBride et al., 1988 [6]
HH 39	Day 13	Bulk tensile test	11 MPa	Kalson et al., 2010 [10]
HH 40	Day 14	Bulk tensile test	20.5 MPa	Kalson et al., 2011 [7]
HH 28– 43	Day 5.5–18	Nanoscale tip indentation	7–21 kPa	Marturano et al., 2013 [8]
HH 28–43	Day 5.5–18	Microscale tip indentation	5–108 kPa	Marturano et al., 2013 [8]
Adult	Adult 8 mo.	Bulk tensile test	210 MPa	Nakagaki et al., 2007 [9]