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## Functional Attachment of Soft Tissues to Bone: Development, Healing, and Tissue Engineering

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### Abstract

Connective tissues such as tendons or ligaments attach to bone across a multitissue interface with spatial gradients in composition, structure, and mechanical properties. These gradients minimize stress concentrations and mediate load transfer between the soft and hard tissues. Given the high incidence of tendon and ligament injuries and the lack of integrative solutions for their repair, interface regeneration remains a significant clinical challenge. This review begins with a description of the developmental processes and the resultant structure-function relationships that translate into the functional grading necessary for stress transfer between soft tissue and bone. It then discusses the interface healing response, with a focus on the influence of mechanical loading and the role of cell-cell interactions. The review continues with a description of current efforts in interface tissue engineering, highlighting key strategies for the regeneration of the soft tissue-to-bone interface, and concludes with a summary of challenges and future directions.

### Keywords

enthesis; insertion site; interface; tendon; ligament; mechanobiology; multiphased scaffold; structure-function; tissue engineering

## INTRODUCTION

The musculoskeletal system functions through the coordinated action of multiple types of tissues. These include connective tissues such as tendon, which joins muscle to bone, and ligament, which connects bone to bone. A specialized interface, called an insertion site or enthesis, integrates tendon or ligament to bone and serves to facilitate joint motion (Figure 1). These interfaces exhibit gradients in tissue organization, composition, and mechanical properties that have several functions, from effectively transferring stress between mechanically dissimilar materials (tendon/ligament and bone) to sustaining the heterotypic cellular communications required for interface function and homeostasis (1–12). A number of common orthopaedic injuries require the repair of a ruptured tendon or ligament to its bony insertion. In the shoulder, rotator cuff tendon ruptures typically necessitate surgical reattachment of the tendon to the humeral head. In the knee, torn cruciate ligaments often require reconstruction using tendon grafts. These critical junctions are not reestablished following surgical repair, with high reinjury rates associated with both procedures.

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Depending on the severity of the injury and the age of the patient, 20–94% of repaired rotator cuff tears fail (13). In the knee, approximately half of anterior cruciate ligament (ACL) reconstruction patients experience knee pain at one year post surgery (14), and failure to regenerate the complex soft tissue–to-bone insertion site compromises graft stability and long-term clinical outcomes (15, 16).

Tendons and ligaments attach to bone via either fibrous or fibrocartilaginous insertions (17). Fibrous insertions typically occur over large areas, presumably to distribute forces and reduce stresses, and are characterized by perforating mineralized collagen fibers (18). For example, the medial collateral ligament attaches to the tibia and the deltoid tendon attaches to the humeral head across fibrous insertions. Fibrocartilaginous insertions are more common and include the bony attachments of the rotator cuff tendons, the ACL, and the Achilles tendon. Because of their clinical relevance with respect to the most common injuries to ligaments (e.g., rupture of the ACL) and shoulder (e.g., torn rotator cuff), fibrocartilaginous insertions are the focus of this review. These insertions are typically narrow (on the order of hundreds of micrometers) and have classically been categorized into four zones (17). The first zone consists of tendon/ligament, is populated by fibroblasts, and has mechanical properties similar to those of the tendon/ligament mid-substance. It is composed of linearly arrayed type I collagen fibers (6, 19). The second zone consists of fibrocartilage, is populated by fibrochondrocytes, and is composed of types I, II, and III collagen and the proteoglycan aggrecan (6, 19–22). The third zone is also populated by fibrochondrocytes and is composed of mineralized fibrocartilage. Here, type II collagen predominates, and there are significant amounts of type X collagen as well as aggrecan (6, 19–22). The fourth zone consists of bone proper, within which osteoblasts, osteocytes, and osteoclasts reside in a matrix of mineralized type I collagen. Although the insertion site has classically been defined as containing these four zones, it is emphasized that the stratified tissue regions are compositionally distinct but structurally continuous. As described in subsequent sections, this controlled spatial distribution in matrix structure and composition is largely responsible for the functional grading necessary for minimizing stress concentrations between the connective tissue and bone.

Due to their functional significance, there exists a growing interest in the healing and regeneration of soft tissue–to-bone attachments. Furthermore, as the field of tissue engineering (23, 24) advances, the next step is to engineer complex tissues with integrated interfaces (5, 25–31). The complexity of the transition between unmineralized and mineralized tissues, however, poses significant challenges for effective interface healing and regeneration, with multiple biological and mechanical factors regulating the development as well as the healing of the soft tissue–to-bone interface. Focusing on tendon-to-bone and ligament-to-bone insertions, this review begins with a discussion of current understanding of enthesis development and maturation, with a closer look at the resultant structure-function relationships at soft tissue–to-bone attachments. Next, the mechanisms underlying the healing of the injured interface, in particular the role of mechanical loading and cellular interactions, are discussed. The final section of the review highlights current tissue engineering efforts for the regeneration of ligament- or tendon-to-bone interfaces, discussing biomimetic scaffold design as well as scaffold- and cell-based strategies for engineering a functional gradient with mechanical properties that approximate those of the native interface. This review concludes with a summary of potential challenges and future directions in an effort to understand and regenerate these critical soft tissue–to-bone attachments.

## THE DEVELOPMENT OF STRUCTURE-FUNCTION RELATIONSHIPS AT THE ENTHESES

### Enthesis Development

During fetal and postnatal development, a complex transitional tissue forms between bone and connective tissues such as tendon or ligament (3, 6, 32–37). Enthesis development is initially driven by endochondral ossification: Cartilage mineralizes to form bone, and a fibrocartilaginous transition then develops at the interfaces between the bone and connective tissues. In situ hybridization and immunohistochemistry studies have revealed the spatial and temporal expression pattern for extracellular matrix components during this developmental process (Figure 2) (3, 6, 32–37). Type I collagen (characteristic of fibroblasts and osteoblasts), type II collagen (characteristic of chondrocytes), and type X collagen (characteristic of hypertrophic chondrocytes) were localized to developing tendon/ligament-to-bone insertions (3, 6). In the mouse shoulder, the fibroblasts of the supraspinatus tendon expressed type I collagen at all fetal and postnatal time points studied. The chondrocytes in the as-yet-unmineralized bone expressed type II collagen postnatally for 14 days, and those near the insertion became hypertrophic at this point and began expressing type X collagen. Mineralization proceeded via endochondral ossification of a subset of the type II collagen-expressing cells, resulting in a mineralized fibrocartilage region adjacent to the unmineralized tissue (Figure 3) (38). After this initial phase of endochondral ossification, the collagen fibers and mineral were remodeled to form a mature, well-organized, transitional tissue between the tendon and bone. Similar patterns were described for rat Achilles tendon insertions and for bovine ACL insertions postnatally and for the deltoid-humeral tuberosity attachment in utero (6, 32, 37).

A number of biologic and mechanical factors drive the development of a transitional tissue at the interface between tendon/ligament and bone (Figure 2). Many of the cellular and molecular events reported during mineralization of the entheses follow pathways similar to those seen during chondrocyte hypertrophy in the growth plate. The hypertrophic chondrocytes of the growth plate mineralize the matrix between the cells, producing a zone of provisional calcification. The factors that modulate growth plate maturation also play a role in entheses formation. Specifically, the Indian hedgehog (Ihh)/parathyroid hormone-related protein (PTHrP) feedback loop is critical for mineralization at the growth plate. The Ihh/PTHrP loop regulates chondrocyte differentiation and homeostasis (39–41) and plays a role in entheses development (37, 42–46). PTHrP, for example, prevents proliferating chondrocytes in the growth plate from becoming hypertrophic chondrocytes (which eventually mineralize) (42). A population of proliferating chondrocytes can therefore be maintained by PTHrP and remain available for growth rather than for hypertrophy and mineralization. At the entheses, graded expression of Ihh and PTHrP may regulate the formation of a graded transition between mineralized and unmineralized tissue.

Two transcription factors necessary for chondrogenesis and tenogenesis, SOX-9 and scleraxis (Scx), respectively, also likely play important roles for tendon/ligament-to-bone development (47). SOX-9 is necessary for chondrogenesis (48), and Scx is necessary for tenogenesis (49–52). Their localized expression patterns define the transition between tendon and fibrocartilage (Figure 2).

Blitz et al. (37) recently examined the development of the deltoid tendon-humeral tuberosity attachment and described the interplay of some of these factors. The authors observed that the deltoid tuberosity formed via endochondral ossification in a two-phased process: Initiation was regulated by a signal from the tendon, whereas the subsequent growth phase was muscle dependent. Specifically, Scx regulated bone morphogenetic protein (BMP)-4

production in tendon cells at the bony insertion site. When BMP-4 expression was blocked in Scx-expressing cells, the enthesis (and associated bone ridges) did not form. It is therefore likely that BMP-4 is a key mediator of tendon-specific signaling for enthesis formation. As in other reports, the key regulators of endochondral ossification (e.g., type II collagen, *Ihh*, PTHrP, type X collagen) were expressed at the developing enthesis. The coordinated and spatially localized expression of these important growth and differentiation regulators drives the formation of the specialized tissue that attaches soft tissues to bone.

### Role of Loading in Enthesis Development

Biophysical cues influence the development of tendons, ligaments, cartilage, and bone (53–56). All of the cell types found along the enthesis are mechanoresponsive; therefore, a role for mechanobiology during the development of this tissue is expected. Studies using genetically modified mice with muscular defects demonstrated that, although muscle loading was not required for initiation of enthesis formation, it was necessary for the subsequent growth and maturation of the enthesis (37). The role of muscle loading in the development of the tendon-to-bone insertion was also examined in a murine shoulder model by Thomopoulos et al. (35, 57) and Kim et al. (58). Rotator cuff muscles were paralyzed using either intramuscular injections of botulinum toxin A or laceration of the upper trunk of the brachial plexus. As the main events driving enthesis development of the rotator cuff occur postnatally, paralysis was induced within 24 hours of birth. Based on assessments of muscle volume, muscle mass, and force generation capacity, both methods of muscle paralysis resulted in decreases in loading across the developing enthesis (59). The reduction in muscle loading impaired mineral deposition and fibrocartilage formation and led to disorganized fiber distribution and inferior mechanical properties at the developing tendon enthesis. The amount of mineral that accumulated in the humeral head decreased when loading was removed (35, 58). Bone volume decreased and bone architecture (e.g., trabecular thickness) changed when comparing saline-injected with botulinum toxin-injected shoulders. Similar results were seen when comparing neurotomy shoulders with normal shoulders. Of particular note is that differences were not apparent at early time points, indicating that, similar to the study by Blitz et al. (37), muscle loading may not be necessary to initiate enthesis development. The lack of bone formation in unloaded insertions was at least in part due to increased levels of bone resorption, as evidenced by increased osteoclast numbers in the paralyzed group compared with the control groups. This was confirmed by the observation that suppression of osteoclast activity partially rescued the defects caused by unloading (60). These results demonstrate that mineralization at the developing enthesis is sensitive to mechanical loading. Fibrocartilage formation was impaired in paralyzed shoulders (35, 58), and a zone of hypertrophic chondrocytes was evident between the tendon and its bony insertion at early postnatal timepoints. Within 21 days, this zone matured into a graded, mineralized insertion in the loaded shoulders and remained disorganized, with little fibrocartilage, in the paralyzed shoulders. Within 56 days, insertions in the botulinum toxin-injected shoulders consisted of disorganized collagen fibers with sparse amounts of fibrocartilage. Consistent with the mineral accumulation results, postnatal removal of mechanical loading cues dramatically affected fibrocartilage formation at the developing enthesis. Decreased muscle loading also led to a decrease in the organization of collagen fibers at the insertion (57). The structural and compositional changes described above resulted in decreased mechanical properties in the unloaded group compared with the loaded group (57). The molecular mechanisms that control the mechanoresponsiveness of the developing enthesis remain unclear, although studies have found that expression of *Ihh* and PTHrP is mechanosensitive (43). In summary, the formation of a functionally graded transition between tendon and bone requires physiologic muscle loading.

## Structure-Function Relationships at the Tendon/Ligament Enthesis

The tightly regulated and highly ordered enthesis developmental process leads to a robust attachment between tendon/ligament and bone. Attachment of a compliant material such as tendon or ligament (with a modulus on the order of 200 MPa) to a relatively stiff material such as bone (with a modulus on the order of 20 GPa) poses unique challenges. For example, stress concentrations would arise at the interface of these two materials if the attachment were not adjusted to alleviate the modulus mismatch. Functional grading is a well-established method to minimize stress concentrations at material interfaces (62). Similarly, the tendon- and ligament-to-bone insertion sites are natural functionally graded structures, exhibiting gradients in mechanical, compositional, and structural properties. Studies examining the supraspinatus tendon-to-bone insertions of rats have demonstrated gradations in properties along the enthesis rather than an abrupt interface between mineralized and unmineralized tissue. Along with a compositional gradient in the extracellular matrix, collagen fibers were less oriented at the insertion compared with the tendon, and mineral content increased monotonically from tendon to bone (Figure 3) (3, 38, 63). Biomechanically, both the elastic and viscous behaviors of the tissue varied from tendon to bone (3).

Direct measurement of mechanical properties at tendon/ligament-to-bone insertions has been difficult owing to the heterogeneity and small length scale of the interface (typically 100  $\mu\text{m}$  to 1 mm in length) (6, 64–66). To address these challenges, the local strain distribution at the ACL-to-bone interface under uniaxial tensile load was measured using ultrasound elastography (7). The strain along the insertion site was observed to be region dependent, with the highest strain found at the ligament mid substance, followed by decreased strain progressing from the fibrocartilage interface to the bone. These regional strain differences suggest an increase in tissue stiffness from ligament to bone. Moffat et al. (8) performed microcompression experiments to determine the compressive properties of the ligament-to-bone insertion. Energy dispersive X-ray analysis was used to determine the mineral content along the insertion. Similar to the elastography findings (7), strain decreased gradually from the fibrocartilage interface to the bone (8). The elastic modulus was twice as high in the mineralized fibrocartilage when compared with the nonmineralized interface region (Figure 3). Similar significant increases in modulus were recently reported for meniscal attachments to bone (67, 68).

The region-dependent gradient in mechanical properties across the insertion was correlated to spatial changes in matrix organization and composition (Figure 3). Of particular importance is the role of mineral in increasing stiffness across the insertion. Higher matrix mineral content has been associated with greater mechanical properties in connective tissues (69–71). Evaluation of the ACL-to-bone insertion site using Fourier transform infrared imaging (FTIR-I) (72) and X-ray analysis (8) revealed an exponential increase in phosphate content progressing from the calcified fibrocartilage interface and then to the bone (Figure 3). Similar monotonic gradients in mineral were measured using Raman spectroscopy across developing supraspinatus tendon-to-bone insertions (38, 63). The increase in elastic modulus, progressing from the nonmineralized to the mineralized fibrocartilage regions, was positively correlated with the presence of calcium phosphate (8). The relationship between mineral content and stiffness was modeled recently by Genin et al. (9) using micromechanical approaches. The model predicted that the experimentally observed increase in mineral across the insertion can provide significant stiffening, but only for concentrations of mineral above a percolation threshold corresponding to formation of a mechanically continuous mineral network within each collagen fiber.

The structural organization of an insertion site is optimized to minimize stress concentrations. Although a monotonic increase in stiffness has been reported at the ACL-to-



bone attachment, many anatomic locations contain interfaces with a compliant zone between unmineralized and mineralized tissues. Studies of the rotator cuff (3, 73, 74), patellar tendon (75), and meniscus (67) in adult animals demonstrated that the modulus near their bony insertions is approximately half that at the soft tissue mid substance. Using scanning acoustic microscopy, Sano et al. (74) localized this compliant region to the unmineralized fibrocartilage of the rabbit rotator cuff enthesis. Recent numerical optimizations have provided a rationale for this counterintuitive attachment system (76). Using a mathematical model of the insertion, Liu et al. (76) showed that stress concentrations can be reduced by a biomimetic grading of material properties. The authors developed an optimization scheme to determine the mechanical properties between an idealized tendon and bone that minimized stress concentrations. The solution for axisymmetric tensile loading revealed that stress concentrations were minimized when the insertion included a region more compliant than either tendon or bone, presumably found at the unmineralized region of the enthesis.

A number of additional strategies at various hierarchical levels have been identified for theoretically achieving effective stress transfer between soft tissues and bone. At the millimeter scale, decreasing the angle of attachment prevents stress singularities at the interface (4, 11, 77) and peak stresses can be reduced by optimization of the gross shape of the insertion (11). At the micrometer scale, interlocking of the tissues through interdigitation (78) increases the toughness of the interface. Grading of transitional tissue, for example, gradations in collagen fiber orientation (4, 9) and mineral content (8, 9, 63), produces a functional grading in mechanical properties across the insertion. The combination of these factors across hierarchical levels predicts the reported variation of stiffness [i.e., a compliant region followed by a stiffer region (3, 67, 68)] over the length of soft tissue-to-bone insertions. Further imaging and biomechanical experiments are necessary, however, to validate these modeling results with native tissue properties.

## HEALING AND REGENERATION OF THE ENTHESES

### Current Knowledge of Interface Healing

In contrast to the well-orchestrated enthesis developmental program, studies in animal models have demonstrated that soft tissue-to-bone healing occurs through formation of fibrovascular scar tissue rather than regeneration of a graded fibrocartilaginous transition (34, 79–81). In cases in which soft tissues were repaired directly to bone surfaces (e.g., for rotator cuff repair), a robust healing response was seen, but the mechanical properties of the repair did not approach physiological levels (34, 82, 83). Using a rat rotator cuff model, Thomopoulos et al. (34) showed that although the structural properties reached two-thirds of their normal levels after eight weeks of healing, the material properties remained an order of magnitude weaker than those of the control group. Histologically, a sharp boundary was evident between the soft tissue and the bone. There was no continuity of collagen fibers across the interface and no apparent gradient in mineral content. A fibrocartilaginous transition was rarely seen; rather, the tissue at the healing interface consisted of disorganized scar tissue with high levels of type III collagen. Silva et al. (84) reported similar results in a canine flexor tendon-to-bone repair model (Figure 4).

In cases in which soft tissues were repaired into bone tunnels (e.g., for ACL reconstruction), a zone of loose fibrovascular tissue typically formed between the tendon and the wall of the bone tunnel (79, 85). Liu et al. (86) examined the morphology and matrix composition of the tendon graft-to-bone interface during healing. Two weeks after reconstruction, the tendon attached to the bone, with scar tissue filling the tendon-to-bone junction. This interface tissue was remodeled into a dense connective tissue matrix within one month, with predominantly fibroblasts present. After six weeks, contraction of the interface was prominent, and significantly less type I collagen was found in the remodeling matrix;

however, type II collagen became detectable. This and other studies demonstrate that surgically juxtaposing tendon and bone does not lead to regeneration of the graded fibrocartilaginous interface (79).

Further complicating the repair of soft tissues to bone is the bone loss that typically occurs following tendon or ligament injury (87–90). Decreased bone mineral density was found at the canine distal phalanx at 10, 21, and 42 days following injury and repair, indicating that bone resorption may be a factor that contributes to the low values of repair-site failure force (90). Similar results were reported in the rat rotator cuff model (91, 92), in which bone mineral density was significantly decreased following tendon injury and repair. A delay between injury and repair resulted in inferior tendon-to-bone healing, owing in part to decreased bone quality.

### **The Role of Cellular Interactions During Interface Healing**

Studies of ACL graft-to-bone healing suggest that cell phenotype and communication at the repair site are significant considerations for enthesis regeneration. Moreover, differentiation of multipotent cells into enthesis-relevant populations has the potential to facilitate healing at the interface. As described above, the healthy tendon/ligament-to-bone insertion consists of a progression of tissue types (tendon/ligament, fibrocartilage, mineralized fibrocartilage, and bone), each exhibiting a characteristic cellular phenotype and matrix composition. Although the cells in either soft tissue or bone have been well characterized, the phenotype of the enthesis fibrochondrocyte is not well defined. Sun et al. (93) compared the response of fibrochondrocytes isolated directly from the ligament-to-bone insertion to those of inner- and outer-ring meniscal fibrochondrocytes, as well as to ligament fibroblasts and articular chondrocytes. Aside from their mineralization potential, the ACL insertion fibrochondrocytes are more similar in growth rate and biosynthesis to articular chondrocytes than to meniscal fibrochondrocytes or ligament fibroblasts. It is likely that communication among the three resident cell populations, namely fibroblasts, fibrochondrocytes, and osteoblasts, is important for interface homeostasis and regeneration.

It has long been observed that although tendon-to-bone healing following ACL reconstruction does not lead to the reestablishment of the graded native insertion, a layer of fibrovascular tissue is formed within the bone tunnel (79, 94, 95). This observation suggests that when trauma or injury to the interface results in nonphysiologic exposure of normally segregated tissue types (e.g., bone and ligament), interactions between the resident cell populations in these tissues (e.g., osteoblasts and fibroblasts) have the potential to initiate and direct an interface repair response. Lu & Jiang (5) proposed a working hypothesis for enthesis regeneration, suggesting that osteoblast-fibroblast interactions can mediate regeneration of an enthesis through heterotypic cellular interactions leading to phenotypic changes or transdifferentiation of osteoblasts and/or fibroblasts. Furthermore, these interactions may promote the differentiation of stem cells or progenitor cells into fibrochondrocytes, leading to the regeneration of a fibrocartilage interface.

Several *in vitro* studies evaluating the role of heterotypic cellular interactions on interface regeneration have been reported (41, 96–98). Coculture and triculture models of interface-relevant cell populations were used to determine the effects of cellular communication on the development of fibrocartilage-specific markers *in vitro*. Wang et al. (98) examined the interaction between osteoblasts and ligament fibroblasts: A two-dimensional (2D) coculture model permitting both cell physical contact and soluble factor interactions was designed to emulate the *in vivo* condition in which the soft tissue graft is in direct contact with bone tissue following ACL reconstruction (Figure 5). Osteoblasts and fibroblasts were first separated by a hydrogel divider, and upon reaching confluence, the divider was removed, allowing the osteoblasts and fibroblasts to migrate and interact directly within the interface

region (Figure 5). It was reported that these controlled interactions decreased cell proliferation (Figure 5), altered the alkaline phosphatase (ALP) activity profile, and promoted the expression of matrix proteins characteristic of the fibrocartilage interface, such as types I and II collagen, and cartilage oligomeric matrix protein (COMP). In addition to physical interaction between cells, subsequent conditioned media studies revealed that both autocrine and paracrine factors were responsible for the changes in phenotype observed during osteoblast-fibroblast coculture (99).

Although osteoblast-fibroblast interactions in coculture resulted in phenotypic changes and the expression of interface-relevant markers, a fibrocartilage-like interface was not formed *in vitro*. Interestingly, after coating tendon grafts with mesenchymal stem cells (MSCs) embedded in a fibrin gel, the formation of a zone of cartilaginous tissue between graft and bone was observed, suggesting a potential role for stem cells in fibrocartilage formation (100). In other words, fibrochondrocyte precursors or stem cells may be involved in interface regeneration, and osteoblast-fibroblast interactions may regulate fibrochondrogenic differentiation of these cells during healing. In addition, fibroblasts from tendon, ligament, and skin have been shown to exhibit fibrochondrocyte- or chondrocyte-like phenotypes under certain conditions (101–105). To test the hypothesis that stem cells may be induced toward a fibrochondrocyte lineage by factors produced from osteoblast-fibroblast interactions, Wang & Lu (106) designed a triculture system of fibroblasts, osteoblasts, and MSCs. The triculture consisted of fibroblasts and osteoblasts each seeded on cover slips on opposite sides of a well with MSCs preloaded into a hydrogel insert. Under the influence of osteoblast-fibroblast interactions, the MSCs exhibited a level of alkaline phosphatase activity similar to that of enthesis fibrochondrocytes, with both groups peaking by day 7 and decreasing thereafter. Moreover, both type II collagen production and a significantly higher level of proteoglycan synthesis were measured for the MSCs in triculture, with values approaching those of insertion fibrochondrocytes.

Findings from the *in vitro* coculture and triculture studies described above (*a*) provide preliminary validation of the hypothesis that osteoblast-fibroblast interactions can induce enthesis-specific markers in MSCs and (*b*) imply a role for heterotypic cellular interactions in regulating the maintenance of soft tissue-to-bone insertions. Although the mechanisms of interaction and the nature of the regulatory soluble factors that are secreted remain elusive, cell-cell communication may play an important role not only in interface regeneration in the healing setting but also in homeostasis in the uninjured setting.

### The Influence of Mechanical Stimuli on Interface Healing

The role of mechanobiology during the healing process is unclear. Although all three components of the insertion site (tendon, fibrocartilage, and bone) are responsive to load when healthy, their response to load during tendon-to-bone healing has required further study. Results from animal models have demonstrated that low levels of controlled load (e.g., via cast immobilization) are optimal for healing (34, 107–109). In a rotator cuff injury-and-repair animal model, cast immobilization was demonstrated to be beneficial for tendon-to-bone healing compared with exercise (34, 107). Similarly, a study comparing early with delayed mechanical loading on tendon-to-bone healing in an ACL model demonstrated that delayed application of cyclic load improved tendon-to-bone healing (as evidenced by biomechanical and histologic assessment) more than immediate loading or prolonged immobilization did. Despite improvements in healing with controlled levels of low load, a multitissue transition was not regenerated in either model; rather, the repair site was characterized by fibrovascular scar tissue. As expected, increased loading stimulated matrix formation, leading to larger cross-sectional area in the high-load groups of the rotator cuff model compared with the cast-immobilized group. However, the orientation of the collagen



fibers approached normal only in the cast-immobilized group. Furthermore, increased loading led to lower mechanical properties. Increased loading was therefore effective in stimulating matrix synthesis but ineffective in improving the mechanical properties of the repair. More material of lesser quality was produced when loading was increased. The beneficial effect of decreased loading may be due in part to a suppression of acute inflammation (109). Immobilization reduced macrophage numbers in an ACL repair model, leading to improvements in mechanical properties and tendon-bone integration. A key feature of the failed healing response in all studies was the lack of a functionally graded transition between the tendon and the bone. Instead, the transition zone was comprised of disorganized scar tissue.

The surprisingly positive outcomes in the cast-immobilized and delayed-loading groups have led to additional studies on the role of mechanical loading on tendon-to-bone healing. It was noted that the supraspinatus muscle in cast-immobilized animals was fully innervated and therefore able to generate load across the repair site in the absence of shoulder motion. The effect of complete removal of load on tendon-to-bone healing has been examined (110, 111), testing the hypothesis that complete removal of load would be detrimental to healing and guide repair. Specifically, removal of load via botulinum toxin paralysis or tenotomy was shown to impair healing (110, 111). Muscle paralysis or tenotomy combined with cast immobilization was detrimental to healing, as evidenced by decreased mechanical properties. The structural properties of the healed tendons were significantly greater in the control specimens compared with the unloaded specimens. These studies showed that when all load is removed from the healing tendon, the cross-sectional area and the structural properties decrease compared with using protective cast-immobilization alone. In conclusion, these studies demonstrate that a fine balance must be reached between excessive loads (which can lead to microdamage) and insufficient loads (which can lead to a catabolic environment) to maximize tendon-to-bone healing.

## CURRENT APPROACHES TO INTERFACE REGENERATION

### Tissue Engineering Strategies

Given the functional importance of the interface and its clinical significance in soft tissue repair, there is substantial interest in regenerating these critical tissue-to-tissue transitions. Utilizing a combination of cells, growth factors, and/or biomaterials, tissue engineering (23, 24) has already been applied to the formation of bone, ligament, and tendon *in vitro* and *in vivo*. Presently, a critical barrier to clinical translation in musculoskeletal tissue engineering is achieving biological fixation or functional integration of these grafts with each other and/or with the existing musculo-skeletal system. This section reviews current tissue engineering strategies aimed at regenerating the tendon-to-bone or ligament-to-bone interfaces, focusing on biomimetic scaffolds as well as cell- and growth factor-based approaches.

The guiding principle of interface tissue engineering is a strategic recapitulation of the complex structure-function relationships inherent in native tissue interfaces. As discussed above, these tissues, each with a distinct cellular population, must operate in unison to facilitate physiologic function and maintain tissue homeostasis. It is thus not surprising that the transitions between tissue types are characterized by high levels of heterogeneous structure and composition that are crucial for musculoskeletal function. In light of the native interface's complexity, strategic biomimicry is critical for interface regeneration and seamless graft integration, with key design parameters selected based on current understanding of interface structure-function relations as well as the mechanism of interface formation, healing, and homeostasis. Specifically, based on the multitissue matrix organization at common tendon- and ligament-to-bone insertions, interface scaffold design must support the deposition of compositionally distinct yet structurally contiguous tissues,

as well as exhibit a gradient of mechanical properties similar to that of the native tissue. Typically, a stratified or multiphased scaffold can be used to capture the multitissue organization observed at the natural interface. To minimize stress concentrations, the stratified scaffold should exhibit phase-specific composition and structural organization, resulting in a gradation of mechanical properties similar to that of the native tissue-to-tissue junction.

Although a stratified scaffold is essential for recapitulating multitissue organization, a key criterion is that these strata or phases are interconnected and preintegrated with each other, thereby supporting the formation of functionally continuous multitissue regions. Moreover, phase-specific or *trans*-phase gradients can be engineered to more closely mimic the complexity of tissue-to-tissue transitions. In particular, given the documented progression from noncalcified to calcified interface regions at both tendon- and ligament-to-bone insertion sites, exercising spatial control over mineral distribution on the scaffold is essential. Compared with a homogeneous structure, a scaffold with predesigned, tissue-specific matrix inhomogeneity can better sustain and transmit the distribution of complex loads inherent at the multitissue interface. Furthermore, interactions between relevant cell population must be regulated and exploited, as cellular interactions play an important role in the formation, homeostasis, and repair of interfacial tissues (41, 96–98). Therefore, control over the spatial distribution of cell populations and biologic factors is another key design parameter to be considered. Finally, for biological fixation, interface regeneration must be coupled with osteointegration. Ultimately, along with engineering compositionally distinct yet structurally continuous multitissue regions, the end goal of interface tissue engineering is to establish a functional gradient of mechanical properties mimicking those of the native insertion site, an accomplishment that is essential for integrative soft tissue repair.

### Ligament-to-Bone Interface Tissue Engineering

The regeneration of a multitissue transition from ligament to bone described above has long posed a significant challenge for functional ligament tissue engineering. Early attempts to improve the fixation of a ligament graft to bone focused on augmenting the surgical graft with a material that would encourage bone tissue ingrowth within the bone tunnel (112–117). For example, when calcium phosphate cement was used to fill the tendon-to-bone junction in a rabbit ACL reconstruction model, the ceramic augmented bone tissue growth and organization (113); similar findings were reported with the injection of tricalcium phosphate cement into bone tunnel (114). An alternative approach to improving tendon osteointegration included soaking ACL reconstruction grafts in a series of solutions that facilitated the formation of a calcium phosphate layer prior to implantation (118). This resulted in direct bonding between the implanted graft and the surrounding bone after three weeks. Other approaches to improve bone tunnel osteointegration include the addition of periosteum wraps to the region of the graft that interacts with bone (119–123) and of growth factors such as BMP-2 (85, 124–127), BMP-7 (128), and granulocyte colony stimulating factor (129). Additionally, the direct application of MSCs for improving graft-to-bone integration has been explored (130–132). Although these methods have enhanced graft integration within the bone tunnel, they do not yet result in the regeneration of the anatomic fibrocartilaginous interface.

Published studies in ACL tissue engineering have centered on regenerating the ligament proper (133–135), with more recent studies focusing on the integration of ACL with bone (136–139). Cooper et al. (137) reported on a multiphased design of a synthetic ACL graft fabricated from three-dimensional braiding of polylactide-coglycolide fibers with a ligament proper as well as two bony regions. In vitro (138) and in vivo (139) evaluation demonstrated biocompatibility and ligament healing in a rabbit model. Using MSCs, Ma et al. (140)

formed bone-ligament-bone constructs by combining cell-engineered bone with ligament monolayers, which resulted in the self-assembly of a ligament-bone-ligament-like construct. Paxton et al. (141) incorporated both HA and RGD peptides in a polyethylene glycol hydrogel in order to engineer ligament-to-bone attachments. These novel ACL designs represent advances over single-phased ACL grafts, and the next step is to tackle the challenge of biological fixation, by incorporating the fibrocartilage interface into ACL graft design.

To this end, Spalazzi et al. (142, 143) developed a triphasic scaffold for the regeneration of the ACL-to-bone interface (Figure 6). Modeled after the native stratified transition, the scaffold consisted of three compositionally distinct yet structurally continuous phases: Phase A (PLGA 10:90 mesh) was designed for fibroblast culture and ligament formation, Phase B (sintered PLGA 85:15 microspheres) was designed as the interface region intended for fibrochondrocyte culture and fibrocartilage formation, and Phase C (sintered PLGA 85:15 and 45S5 bioactive glass composite microspheres) was designed for bone formation (144). In addition to supporting the formation of ligament, interface, and bone, predesigned structural and compositional differences between phases enabled compressive mechanical properties to increase across the stratified scaffold, with the highest elastic modulus and yield strength in Phase C (doubling those of Phase B), effectively mimicking the functional grading of the native enthesis. To form the ligament and bone regions, fibroblasts and osteoblasts were seeded onto Phase A and Phase C, respectively (Figure 6). In vitro and in vivo evaluations (142, 143) revealed extensive tissue infiltration and abundant matrix deposition on Phase A and Phase C with coculture, with tissue continuity maintained across all three scaffold phases. Interestingly, matrix production compensated for the decrease in mechanical properties accompanying scaffold degradation, and the phase-specific matrix heterogeneity was maintained in vivo (143).

For fibrocartilage interface formation, Spalazzi et al. (143) extended their in vivo evaluation of the stratified scaffold to triculture, seeding chondrocytes along with fibroblasts and osteoblasts on their respective tissue phases. Specifically, chondrocytes were loaded via a hydrogel carrier into Phase B, and fibroblasts and osteoblasts were preseeded onto Phase A and Phase C, respectively. At eight weeks, an extensive and contiguous collagen-rich matrix was observed across the scaffold phases (Figure 6). In addition to ligament-like matrix in Phase A and bone-like matrix in Phase C, a fibrocartilaginous tissue with chondrocyte-like cells in a matrix of types I and II collagen and glycosaminoglycans was observed in Phase B, with both cell shape and matrix morphology resembling that of the neonatal ligament-to-bone interface (6).

For clinical application, the triphasic scaffold can be used as a graft collar to guide the reestablishment of an anatomic interface directly on ACL reconstruction grafts. The feasibility of such an approach was demonstrated with a mechanoactive scaffold based on a composite of PLGA 85:15 nanofibers and sintered microspheres (145): Scaffold-induced graft compression resulted in significant matrix remodeling and the upregulation of fibrocartilage interface-related markers such as type II collagen, aggrecan, and transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3). These promising results demonstrate that biomimetic stratified scaffold design coupled with spatial control over the distribution of interface-relevant cell populations can lead to interface regeneration. A key criterion to the success of these stratified scaffolds is that the strata or phases are interconnected in order to avoid delamination (146, 147), thereby enabling the scaffolds to support the formation of continuous multitissue regions.

## Tendon-to-Bone Interface Tissue Engineering

Similar to the ligament-to-bone interface, the tendon-to-bone interface exhibits a zonal distribution of extracellular matrix components (Figure 1) (1, 148). Despite similarities, the biological fixation strategies for tendon-to-bone repair should be adapted to accommodate the specifics of the loading environment, mineral distribution, and surgical repair methods for the particular anatomic location.

Several groups have evaluated the feasibility of integrating tendon with bone or biomaterials through reformation of the enthesis. For example, periosteum (149) and demineralized bone matrix (DBM) (150) have been used for interface regeneration. After the periosteal flap was sutured to the end of a torn rabbit infraspinatus tendon and then attached to bone, a fibrocartilage-like matrix developed after 12 weeks, and failure load increased significantly over time. To harness the osteogenic and chondrogenic potential of DBM, Sundar et al. (150) interposed DBM between patellar tendon and osteotomized bone in an ovine model and found that DBM-augmented repair significantly improved weight bearing and fibrocartilage deposition.

The delivery of biological factors during healing has also been explored to improve tendon-to-bone integration. These have included growth factors (151–153) and matrix metalloproteinase (MMP) inhibitors (154, 155). Two recent studies demonstrated that TGF- $\beta$ 3 may accelerate healing (152, 153). Manning et al. (153) used TGF- $\beta$ 3 release to encourage tendon-to-bone healing in a rat rotator cuff repair model, which resulted in increased cellularity, vascularity, inflammation, and cell proliferation post repair. Improvements in mechanical properties were also observed compared with controls. Additionally, Rodeo et al. (151) examined the effect of a mixture of osteoinductive factors on tendon-to-bone healing in an ovine infraspinatus tendon model. Abundant formation of new bone, fibrocartilage, and soft tissue was observed, accompanied by an increase in tendon attachment strength. Recently, the influence of MMP inhibition (155) on tendon-to-bone healing was also examined in a rat model, in which the application of recombinant  $\alpha$ -2-macroglobulin (A2M) protein (a universal MMP inhibitor) to the repaired supraspinatus tendon-to-bone interface increased fibrocartilage formation, improved collagen organization, and reduced collagen degradation.

Cell-based approaches have also been explored for enhanced tendon-to-bone healing. When Gulotta et al. (156) delivered MSCs to the cuff repair site in rats, no improvements in healing were found. Interestingly, positive results were observed after MSCs were transfected with Scx, a transcription factor critical for tendon development (157). Groups receiving Scx-transfected MSCs measured higher strength and stiffness compared with the nontransfected MSC repairs. In a similar study, MSCs transfected with membrane type 1 MMP (MT1-MMP), a factor which is upregulated during embryogenesis at tendon-to-bone insertion sites (154), showed significant improvements in healing compared with controls. Higher fibrocartilage production, as well as improvements in mechanical properties, was noted at the repair site. This approach may be further enhanced in future studies using multiple cell types delivered in a spatially graded fashion (158).

Synthetic biomaterials have also been investigated for tendon-to-bone integration. Implantation of a polyglycolide fiber mesh in a rat model led to the formation of an organized fibrovascular matrix at the infraspinatus tendon-to-bone junction (159). Recently, nanofiber scaffolds have been explored for tendon-to-bone interface tissue engineering, largely owing to their biomimetic potential and physiological relevance. These scaffolds can be tailored to match the native tendon matrix, with controlled alignment, high surface area-to-volume ratio, permeability, and porosity (160–164). Scaffold structural properties such as fiber diameter and alignment have been shown to regulate the response of tendon fibroblasts

(165) and MSCs (166). In order to investigate the potential of nanofiber scaffolds for tendon tissue engineering, Moffat et al. (167) evaluated the effects of PLGA nanofiber organization (aligned versus unaligned) on human tendon fibroblast attachment and biosynthesis. Nanofiber alignment was found to be the primary factor guiding tendon fibroblast morphology, alignment, and integrin expression. Fibroblasts synthesized types I and III collagen, the dominant collagen types of the supraspinatus tendon, on nanofiber scaffolds, and collagen deposition was controlled by the underlying fiber orientation. Furthermore, scaffold tensile mechanical properties, directly related to fiber alignment, decreased as the polymer degraded but remained within range of those reported for the native supraspinatus tendon (168). Building upon these promising results, Moffat et al. (169) designed a stratified, composite nanofiber system consisting of distinct yet continuous noncalcified and calcified regions that mimic the organization of native tendon-to-bone insertion sites. The biphasic scaffold was produced by electrospinning; Phase A was comprised of aligned PLGA nanofibers to support the regeneration of the nonmineralized fibrocartilage region, and Phase B was based on aligned PLGA nanofibers embedded with nanoparticles of hydroxyapatite (PLGA-HA) to support the regeneration of the mineralized fibrocartilage region. The biphasic scaffold design was evaluated both *in vitro* (170) and *in vivo* (171), and chondrocyte-mediated fibrocartilage-like extracellular matrix was found in each scaffold phase. Furthermore, mineral distribution was maintained, with calcified fibrocartilage formed on Phase B, which will enable the biphasic scaffold to better integrate with the surrounding bone tissue *in vivo*.

Nanofiber-based scaffolds with gradients of mineral distribution have also been investigated for tendon-to-bone interface regeneration. Li et al. (172) formed a linear gradient of calcium phosphate on PLGA and poly- $\epsilon$ -caprolactone (PCL) nanofiber scaffolds by varying immersion time in concentrated simulated body fluid. Importantly, the mineral gradient imparted a gradation in mechanical properties along the length of the scaffold, with lower strains and higher elastic modulus corresponding to areas of higher calcium-phosphate concentration. In an alternative approach, collagen scaffolds with a compositional gradient of retroviral coating for the transcription factor RUNX2 induced fibroblasts to produce a gradient of mineralized matrix both *in vitro* and *in vivo* (173). Although the spatial resolution of these linear gradients is not yet within physiological range, these scaffold systems hold significant promise for biomimetic tendon-to-bone interface regeneration (174–176).

In summary, current strategies in tendon- and ligament-to-bone interface tissue engineering first tackle the difficult problem of soft tissue-to-bone integration *ex vivo* by pre-engineering the multitissue interface through multiphase scaffold design and then focus on applying the engineered constructs in the *in vivo* tendon-to-bone healing setting. Functional and integrative repair of tendon or ligaments may be achieved by coupling both cell-based and scaffold-based approaches and by recapitulating the complex nano- through macroscale organization of the native interface.

## SUMMARY AND FUTURE DIRECTIONS

The objective of this review was to describe the current understanding of soft tissue-to-bone interfaces (tendon-to-bone and ligament-to-bone), with a focus on interface development, structure function, healing, and regeneration. The native insertion site is a functionally graded tissue, populated by multiple cell types and extracellular matrix components, which develops through a series of coordinated events that are driven by biologic and mechanical factors. Formation of a graded interface between bone and tendon or ligament is initiated by transcription factors that are specific to the various cell types at the attachment. Maturation and growth of the enthesis is then regulated by mechanical loading cues from muscle forces.



Interface-relevant cell populations likely play important roles in interface repair and homeostasis. Current repairs of tendon or ligament injuries rely largely on mechanical fixation and fail to promote regeneration of the well-organized, native multitissue transition between soft tissue and bone.

Scaffold design for interface tissue engineering seeks to recapitulate the structure and function found in the native tissue; a gradation of scaffold structure, composition, and mechanical properties may promote regeneration of the interface. Therefore, stratified scaffolds have been designed with spatial control over heterotypic cell interactions to support the formation of integrated multitissue systems. These novel scaffolds can be further refined by incorporating compositional, structural, and growth factor gradients within or across phases, as well as through the use of biochemical and biomechanical stimulation. Furthermore, functional and integrative soft tissue repair may be achieved by coupling cell-, growth factor-, and scaffold-based approaches.

A number of challenges remain before these tissue engineering approaches can be applied clinically. These include the need for a greater understanding of the structure-function relationships inherent at native tissue-to-tissue interfaces as well as the mechanisms governing interface development and regeneration. There likely exists a cell type at the developing enthesis that is phenotypically distinct from fibroblasts, chondrocytes, and osteoblasts. Although many of the factors seen during endochondral ossification are also expressed during enthesis development, the manner in which an unmineralized tissue is maintained adjacent to the mineralizing cartilage remains unknown. A better understanding of the developmental process may provide targets for enhanced healing of connective tissue-to-bone interfaces or for regeneration of the native insertion. Furthermore, a better understanding is needed regarding the effects of biological, chemical, and mechanical stimulation on interface regeneration *in vitro* and *in vivo*. Tissue-engineered constructs must also be evaluated in physiologically relevant *in vivo* models (under healthy and degenerative conditions) to determine the clinical potential of the designed scaffolds. Finally, regulatory, medical, and ethical challenges related to engineering complex tissues must be considered if clinical translation is to be realized.

In summary, soft tissue-to-bone interfaces play a critical role in musculoskeletal function, and their regeneration via interface tissue engineering represents a promising strategy for improved clinical outcomes. Future studies expanding our understanding of the development, structure-function, and healing of attachments will allow for clinical translation of these regeneration strategies to enable functional and integrative repair of tendon and ligament injuries.

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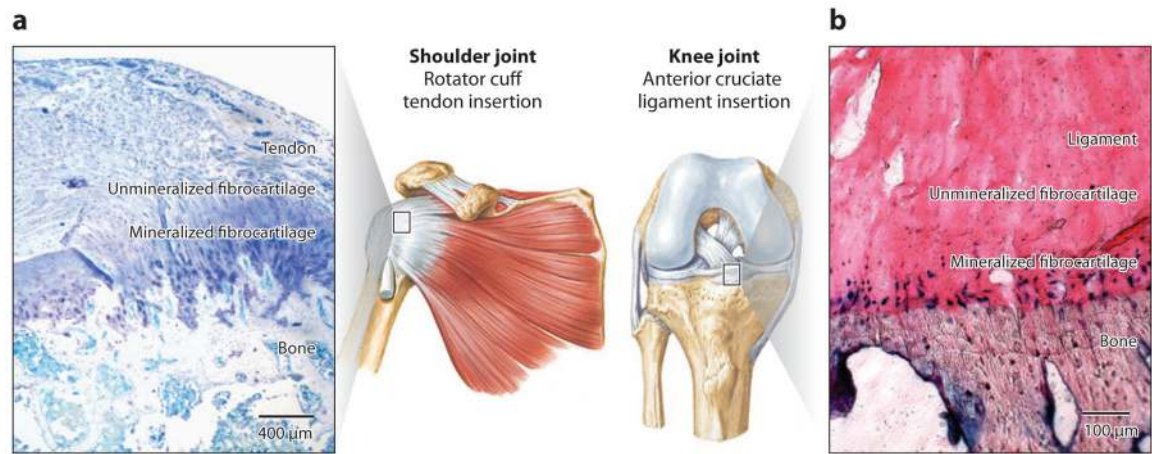
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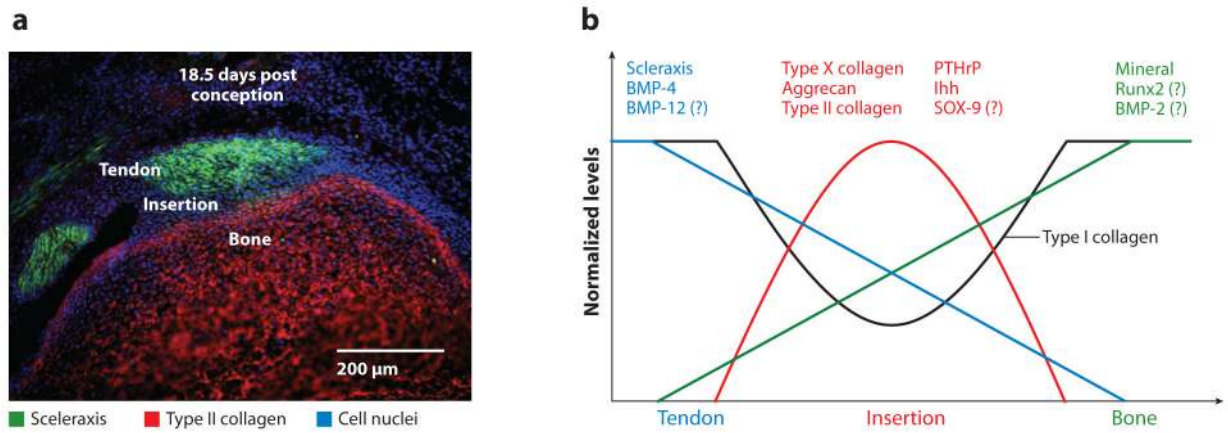
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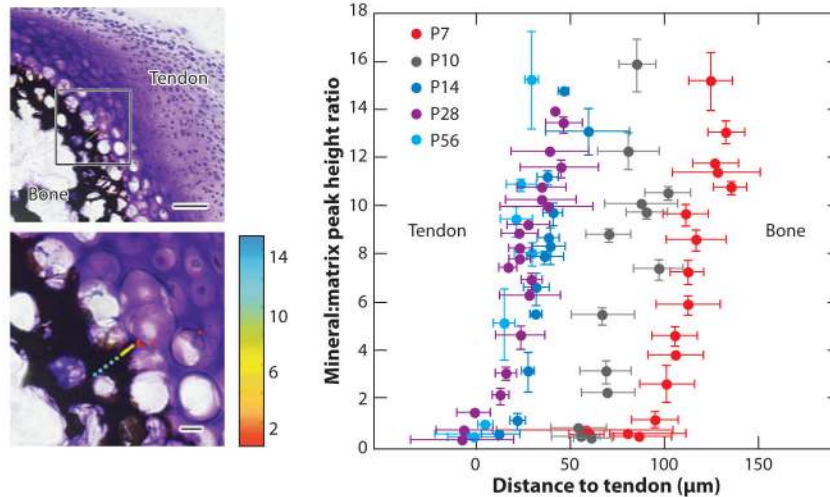
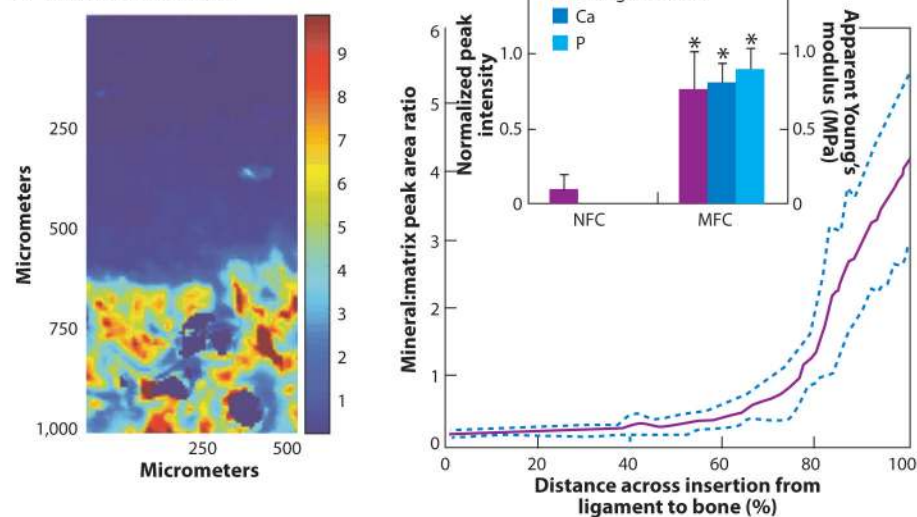


**Figure 1.** (a) Tendon and (b) ligament attach to bone across a functionally graded fibrocartilaginous transition site (a toluidine blue–stained section from an adult rat supraspinatus tendon–to–bone insertion is shown in panel *a*, and fast blue–stained section from a mature bovine anterior cruciate ligament insertion is shown in panel *b*) (3, 6).

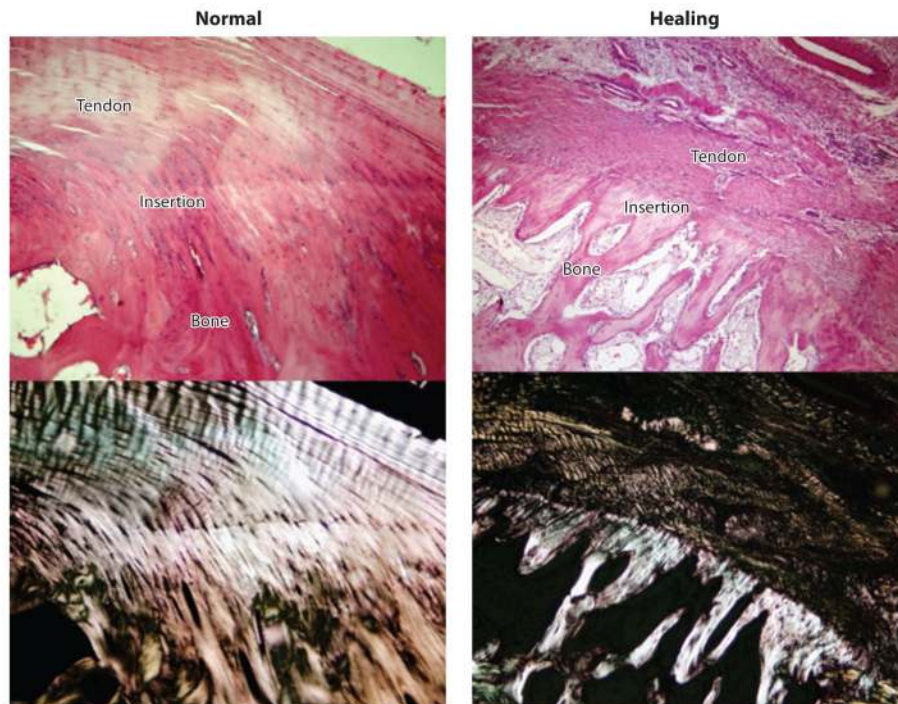


**Figure 2.**

(a) At 18.5 days post conception, scleraxis expression is localized to the supraspinatus tendon and type II collagen expression is localized to the humeral head. A zone of cells at the insertion does not express either marker. (b) Spatially and temporally controlled expression of a number of transcription factors and growth factors drives enthesis development. A question mark (?) indicates that a role for the molecule is expected but has not yet been shown definitively. Abbreviations: BMP, bone morphogenetic protein; Ihh, Indian hedgehog protein; PTHrP, parathyroid hormone-related protein.

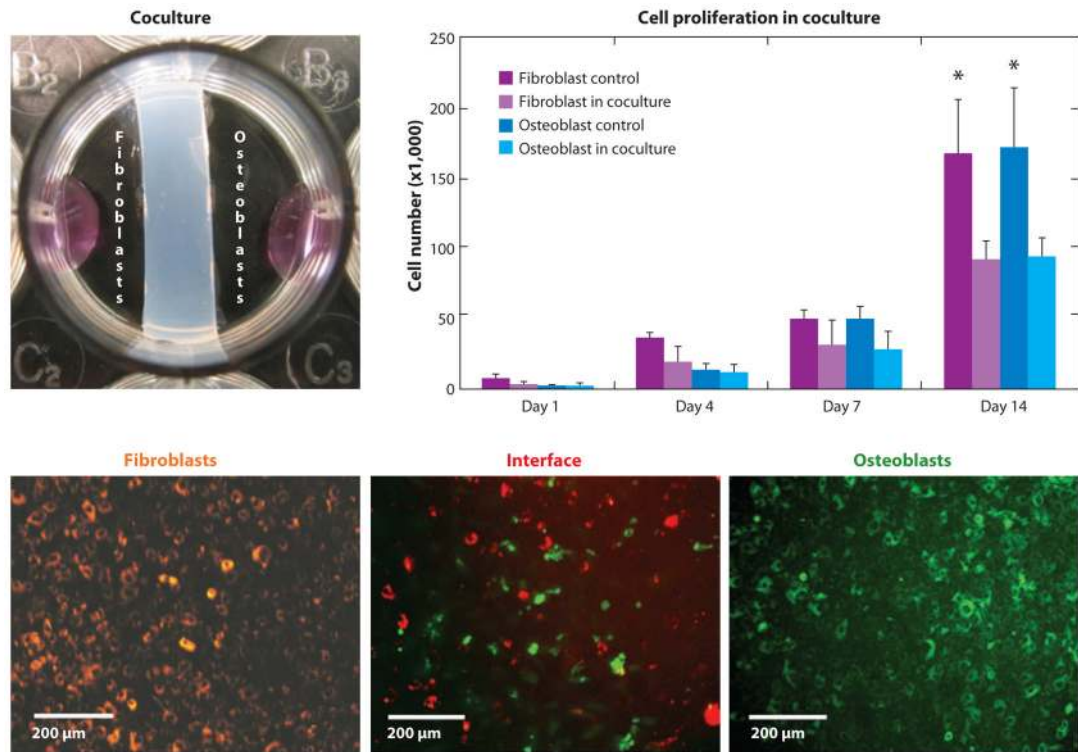
**a** Supraspinatus tendon humeral head insertion**b** ACL tibial insertion**Figure 3.**

(a, left) Spatial gradients in mineral form between supraspinatus tendon and bone at the developing enthesis from the onset of endochondral ossification (age P7 in the mouse, determined via Raman spectroscopy, scale bars = 50  $\mu\text{m}$  for upper left image and 10  $\mu\text{m}$  for lower left image) (38). (right) The mineral gradient migrates from the center of the humeral head at P7 to the tendon attachment by P14. (b) Mineral distribution at the ACL-to-bone insertion increased exponentially across the calcified fibrocartilage region (neonatal bovine, determined via Fourier transform infrared imaging) (72). The significantly higher Ca and P content (\*:  $p < 0.05$ ) in the mineralized fibrocartilage (MFC) versus the nonmineralized fibrocartilage (NFC) region is accompanied by a significant increase in Young's modulus.



**Figure 4.** The functionally graded transition between tendon and bone is not regenerated during the healing process (hematoxylin- and eosin-stained images are shown under bright field in the top row and under polarized light in the bottom row) (84).

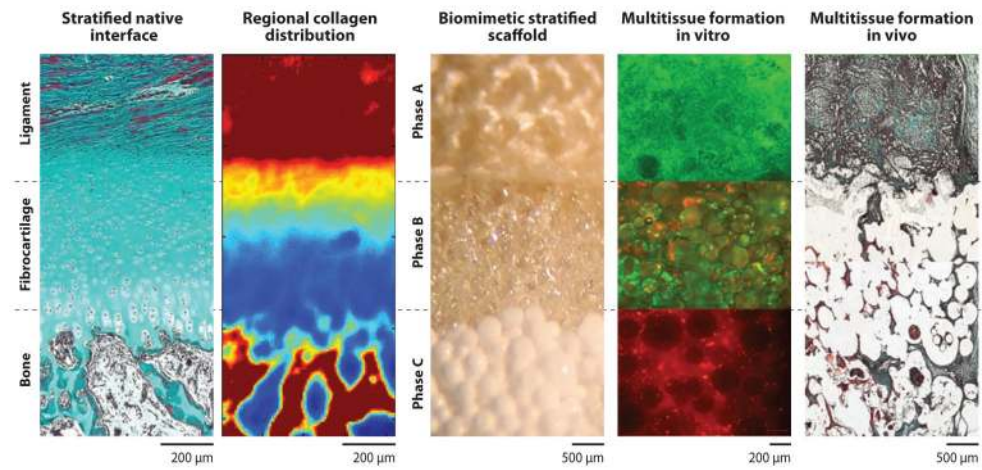




**Figure 5.**

Coculture of fibroblasts and osteoblasts (*upper left*) exerts spatial control of cell distribution, resulting in an interface region that contains interacting osteoblasts and fibroblasts. Fibroblast and osteoblast interactions in coculture reduced cell proliferation (\*:  $p < 0.05$  versus control) (98).





**Figure 6.**

Biomimetic strategy for engineering a ligament-to-bone interface. Inspired by the native enthesis, a stratified scaffold is designed to mimic the layered tissue regions progressing from ligament to fibrocartilage to bone. Spatial control over cell distribution (fibroblasts, chondrocytes, and osteoblasts) on the triphasic scaffold resulted in the formation of compositionally distinct yet structurally continuous tissue regions mimicking those found at the native ligament-to-bone insertion site (27).