Assembly, maturation, and degradation of the supraspinatus enthesis

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The development of the rotator cuff enthesis is still poorly understood. The processes in the early and late developmental steps are gradually elucidated, but it is still unclear how cell activities are coordinated during development and maturation of the structured enthesis. This review summarizes current knowledge about development and age-related degradation of the supraspinatus enthesis. Healing and repair of an injured and degenerated supraspinatus enthesis also remain a challenge, as the original graded transitional tissue of the fibrocartilaginous insertion is not re-created after the tendon is surgically reattached to bone. Instead, mechanically inferior and disorganized tissue forms at the healing site because of scar tissue formation. Consequently, the enthesis never reaches mechanical properties comparable to those of the native enthesis. So far, no novel biologic healing approach has been successful in enhancing healing of the injured enthesis. The results revealed in this review imply the need for further research to pave the way for better treatment of patients with rotator cuff disorder.

\textbf{Level of evidence:} Narrative Review

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\textbf{Keywords:} Rotator cuff; footprint; fibrocartilage; cytokine; mineralization; healing; inflammation

Rotator cuff (RC) lesions are one of the most common conditions affecting the shoulder. The prevalence of RC tears is age dependent, and the prevalence of both partial- and full-thickness RC tears increases markedly after 50 years of age.\textsuperscript{51,83} The etiology of RC diseases is multifactorial, but the supraspinatus (SS) tendon is particularly vulnerable to become injured.\textsuperscript{8}

Longitudinal data suggest that tears progress over time.\textsuperscript{54} Approximately 30\% of surgical repairs will fail, and the failure rate is as high as 90\% in patients with large chronic tears.\textsuperscript{21,74}

The attachment site of the SS tendon at the footprint of the greater tuberosity of the humerus defines a classic enthesis made of 4 zones (Fig. 1). Degenerative changes may be seen in all 4 zones, and the severity of a lesion depends on the degenerative status of the enthesis and the adjacent tissues involved. The SS tendon makes up the roof of the shoulder joint, with the joint side of the tendon being covered by synovial tissue and the acromial side by bursal tissue.\textsuperscript{8,46} It is believed that damage to the enthesis can induce an
inflammatory reaction in the synovium or bursal tissue characterized by expression of matrix-degrading molecules and increased proliferation and differentiation of osteoclasts. This may cause excessive degradation of the enthesis matrix, leading to inadequate enthesis function. The induced imbalance in the enthesis remodeling process is a crucial event in the onset of RC tears and pathologic RC disease.

Both the development and maturation of the native enthesis are poorly understood. Muscle loading (ie, exertion of force on tendon and bone by muscles) is critical for maturation of the developing enthesis, as tissue deformity and diminished mineralization are apparent in mouse models where 1 shoulder has been paralyzed. Besides muscle loading, molecular factors are critical for the development of a functional enthesis. Recently, several transcription factors, including scleraxis (Scx), SRY-box 9 (Sox9), and GLI-Kruppel family member 1 (Gli1), have been detected in early progenitor cells of the developing enthesis and are crucial for development. However, many questions remain unanswered as to what drives development and differentiation of the progenitor cells and how this is regulated.

The reparative capacity of the damaged enthesis is inadequate because the repaired enthesis never regenerates its native structure after an SS tendon tear. Instead, excessive scar tissue formation with inadequate biomechanical properties appears at the repair site. Attempts to enhance the healing process of damaged RC tendons have thus been directed toward biologic enhancement at the repair site, just as stimulation of the regenerative repair processes also has attracted attention. Transforming growth factor β (TGF-β) isoforms have received special attention as important mediators in both scarless and scar-mediated healing processes.

This review provides an overview of recent knowledge on the developmental steps of the fibrocartilaginous enthesis, the inflammatory response after SS tear, and the early characteristics of enthesis healing after damage, touching on therapeutic applications and considerations about biologic augmentation.

Methods

A comprehensive search for peer-reviewed articles, excluding conference papers or reports, was conducted on the basis of the following MeSH terms: enthesis, rotator cuff, supraspinatus, growth and development, inflammation, scleraxis, SRY-box 9, Indian hedgehog, transforming growth factor β, arthritis, tendinopathy, osteoclastogenesis, tumor necrosis factor, synovial, synovium, RANK, and healing.

The following databases were searched for literature: PubMed, Embase, and Web of Science. The reference section of each article was also inspected to find additional articles. The process yielded 85 articles published from 1986 to September 2017.

Structure and function of the native enthesis

The native SS tendon enthesis develops postnatally and is viewed as a 4-zone structure at maturity (Fig. 1). The tendinous first zone...
The supraspinatus enthesis

Table I Time frame of the developing enthesis

<table>
<thead>
<tr>
<th>Progress</th>
<th>Time</th>
<th>Species and model</th>
</tr>
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<tbody>
<tr>
<td>Appearance of scx+/sox9– progenitor cell line</td>
<td>E11.5</td>
<td>Mouse, Sox9+/–; Ail4 and R26R</td>
</tr>
<tr>
<td>Distinct SS tendon appears</td>
<td>E13.5</td>
<td>Mouse, Scx–/–, KO</td>
</tr>
<tr>
<td>TGF-β3 expression in the developing enthesis</td>
<td>E13.5-15.5</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Shift from TGF-β3 to TGF-β1 expression</td>
<td>E15.5</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>TGF-β1 expression in the developing enthesis</td>
<td>E15.5-18.5</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Cells in the SS tendon become increasingly</td>
<td>E18.5</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>more spindle shaped and oriented with the</td>
<td></td>
<td></td>
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<tr>
<td>tendon</td>
<td></td>
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<tr>
<td>Two populations of Hh-responsive cells are</td>
<td>P8</td>
<td>Mouse, ScxCre;Smo6/+/58</td>
</tr>
<tr>
<td>evident</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial mineralization</td>
<td>P7-14</td>
<td>Mouse, Col1/2/10 triple TG</td>
</tr>
<tr>
<td>Fibrocartilage appears</td>
<td></td>
<td>Col3.6-cyan:ColX-cherry double TG</td>
</tr>
<tr>
<td>Collagen type 1– and type 2–expressing</td>
<td></td>
<td>Mouse, ScxCre;Smo6/−/38</td>
</tr>
<tr>
<td>fibrochondrocytes at the base of the enthesis</td>
<td></td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Type X collagen expression occurs</td>
<td>P14</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Hh-responsive cells span the whole developing</td>
<td>P15</td>
<td>Mouse, ScxCre;Smo6/+/58</td>
</tr>
<tr>
<td>enthesis</td>
<td>P21-28</td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Early maturity of enthesis with appearance of</td>
<td></td>
<td>Mouse, ScxCre;Smo6/+/58</td>
</tr>
<tr>
<td>a modest 4-zone structure</td>
<td></td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Mineralized humeral head</td>
<td></td>
<td>Mouse, CD−/−</td>
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<tr>
<td>Fibrocartilage is fully mineralized</td>
<td></td>
<td>Mouse, CD−/−</td>
</tr>
<tr>
<td>Hh-responsive cells are restricted to the</td>
<td>P56</td>
<td>Mouse, ScxCre;Smo6/+/58</td>
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<tr>
<td>unmineralized area</td>
<td></td>
<td>Mouse, CD−/−</td>
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<tr>
<td>Enthesis is fully mature with classical 4-zone</td>
<td></td>
<td>Mouse, CD−/−</td>
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<tr>
<td>structure</td>
<td></td>
<td>Mouse, CD−/−</td>
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<tr>
<td>Hypertrophic chondrocytes are absent from the</td>
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<td>Mouse, CD−/−</td>
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<tr>
<td>mature enthesis</td>
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SS, supraspinatus; TGF, transforming growth factor; Hh, Hedgehog; E, embryonic; P, postnatally; KO, knockout.

is composed primarily of type 1 collagen, with the proteoglycans decorin and biglycan present. This zone is equivalent to the tendon and is populated by tendon fibroblasts. The uncalcified fibrocartilage of the second zone consists of collagen types 1 and 2 with aggrecan, produced by resident fibrochondrocytes. Calcification of the fibrocartilage occurs in the third zone of mineralized fibrocartilage. This represents the actual transition of noncalcified to calcified tissue, demarcated by a clear straight line called the landmark (Fig. 1). It consists of hypertrophic fibrochondrocytes producing collagen type 2, collagen type X, aggrecan, and alkaline phosphatase (AP). Collagen type X is believed to stimulate tissue mineralization. During maturity of the interface, the loss of hypertrophic chondrocytes leads to increased mineralization density, and although unexpected, collagen type X is still present. The fourth zone consists of dense collagen type 1 bone (Fig. 1).

By histologic analysis of mouse SS tendons obtained from different embryonic (E) and postnatal (P) periods, Galatz et al identified a transition zone between the SS tendon and the humeral head at P7, with a visible 4-zone transition at P21 and P28 (Table I). The fully mature interface was present at P56. In support of this, Schwartz et al found a graded mineralization in the calcified zone with the presence of hypertrophic chondrocytes in P7 mice. Using micro–computed tomography scanning, they also observed a mineralization front adjacent to the SS tendon at P14 and an increase in bone mineral density over time. A 4-zone transition with mineralized fibrocartilage was visible at P28. Interestingly, they also noticed that the mineralization was located intercellularly but did not always surround the cells. Thus, mineralization in some instances occurred on only 1 side of the cells. This observation indicates either that the cells are able to control mineralization or that mineralization occurs by transportation and deposition of calcium by cells located away from the interface. The mineralized fibrocartilage of the enthesis does not become reabsorbed, as observed for bone formation. Instead, the mineralized fibrocartilage is maintained throughout life, and so it does not become vascularized.

Developmental gene expression

Temporal expression of a number of genes has been linked to the successful and functional development of the native enthesis. The earliest progenitor cells found in the transitional zone express both Scx and Sox9 (Scx+/Sox9− cells). These cells also express growth and differentiation factor 5 (GDF5), also known as bone morphogenetic protein 14 (BMP-14) or cartilage-derived morphogenetic protein 1 (CDMP-1), during the early stage of development.

Both Scx and Sox9 are crucial for the formation of a proper functional enthesis. Scx is required for the development of tenocytes in force-transmitting tendons and for matrix organization. Sox9 is required for chondrogenic condensation and thus the formation of chondrocytes and primary cartilage, the template, in which the long bones develop. Scx knockout mice display decreased bone mineral density with age, decreased enthesis attachment strength, more disordered orientation of collagen fibers, and loss of cellular morphology gradient during development. This demonstrates the detrimental effect of Scx loss and the necessity of this gene in proper enthesis formation. Also, this suggests that Scx is required for formation of bone eminences, which develop during the embryonic development.
phase on the cartilaginous template of the bone and provide stable footholds for the enthesis. The eminence develops from a pool of Scx̂/Sox̂ cells, directed from the progenitor cells that give rise to the primary cartilage of developing bones by TGF-β. The initial differentiation of these cells is regulated by expression of BMP-4 from cells at the insertional end of the developing tendon. BMP-4 induces differentiation of Scx̂/Sox̂ cells into chondrocytes and directs the formation of bone eminences, forming the initial attachment between tendon and bone, the immature enthesis.

Knockout of TGF-β2/3 or of the TGF-β2 receptor in tendon progenitor cells results in a sudden decrease in Scx expression between E11.5 and E12.5 and eventually a complete loss of expression in the developing tendon. No change in proliferation or cell death is found between E11.5 and E13.5, suggesting a role for TGF-β in the differentiation and maintenance of tenocytes and in the specification of the eminence progenitor cells.

Progenitor cells at the enthesis insertion site express Hedgehog (Hh) proteins. Binding of Hh to its receptor patched 1 (Ptch1) prevents it from inhibiting the transmembrane protein smoothen (Smoo). Active Smoo initiates a signaling cascade activating a transcription factor of the family GLI-Kruppel, which then translocates to the nucleus to initiate Hh gene transcription, such as Gli1 and Ptch1. Without Hh ligand, Smoo remains inactive through the inhibitory effect of Ptch1, resulting in phosphorylation and proteolytic cleavage of Gli, converting it to a transcriptional repressor. By a lineage-tracing experiment, labeling the Hh-specific gene Gli1, researchers were able to locate and follow Hh-responsive cells during SS enthesis development. The results demonstrated Hh-responsive cells as early as E16.5, which located between the cartilage and tendon of the interface and proliferated and stacked in columns between fiber bundles before mineralization, thereby contributing to enthesis growth.

The Hh-responsive cells first spanned the entire fibrocartilage but were restricted to the nonmineralized fibrocartilage at later stages of postnatal development, as they differentiated from nonmineralizing fibrochondrocytes into mineralizing, hypertrophic fibrochondrocytes. All the mature fibrocartilaginous cells were found to be derived from these Hh-responsive cells. As the progenitor cells differentiate, they change collagen expression from only collagen type 1, to collagen types 1 and 2, to only collagen type 2, and finally to collagen type X. When the cells begin to mineralize, they express both collagen type X and AP. The mineralization process starts from the cells closest to the primary cartilage and proceeds toward the tendon midsubstance, as the cells differentiate. Furthermore, the Hh protein expressed in the developing fibrocartilaginous enthesis has been shown to be Indian hedgehog (Ihh). Elimination of Ihh-responsive cells, early in postnatal development, results in reduced mineralization volume and reduced biomechanical properties. Furthermore, inactivation of the Ihh signaling pathway, Smo deletion, leads to reduced expression of extracellular matrix component genes, such as collagen type 2, tenascin C, and lumican, and mineralization genes, such as glypic 3 and asporin. Also, activation of Ihh signaling in tenocytes caused abnormal collagen type 2, tenascin C, and biglycan upregulation in the tendon midsubstance. Studies also showed a lack of tidemark, with reduced differentiation and a significant decrease in proteoglycan content, mineralization, and biomechanical properties at later stages during postnatal development.

However, the collagen fiber organization was maintained overall parallel and organized for both the nonmineralized and mineralized fibrocartilage, as in the tendon midsubstance, but was irregularly shaped and coalesced at the tidemark of the enthesis. Lack of mineralization and irregular collagen fibers at the insertion presumably were the cause of increased insertion strain and reduced biomechanical properties. These results indicate a role for Ihh signaling in controlling and regulating cellular differentiation and maturation of the enthesis fibrocartilaginous zones. In endochondral bone ossification, Ihh is expressed by pre-hypertrophic chondrocytes and stimulates the expression of parathyroid hormone-related peptide (PTHrP). Together, Ihh and PTHrP form a negative feedback loop that regulates chondrogenic differentiation, from pre-hypertrophic to hypertrophic chondrocytes, as well as the rate of cell proliferation. Wang et al demonstrated that the development of the fibrous enthesis is regulated by expression of PTHrP, and deletion of this gene resulted in development of fibrocartilaginous tuberosities and fibrochondrocytes at the fibrous insertion. It is, however, still uncertain whether Ihh also functions to some extent together with PTHrP in the development of the fibrocartilaginous enthesis. A study by Amano et al found the Ihh-specific transcription factors Gli1 and Gli2 to directly bind to and increase the activity of the promoter for collagen type X. In addition, they found Ihh to induce expression of the hypertrophic transcription factor runt-related transcription factor 2 (Runx2) and AP. Furthermore, they found the presence of a transcriptional complex on the collagen X promoter, consisting of Gli1/2, Runx2, and Smads of the BMP-2 pathway. BMP signaling has previously been correlated to the proliferative regulation of chondrocytes and found to regulate hypertrophic differentiation through Smad activation.

TGF-β is important for differentiation and growth during skeletal connective tissue development but also for inflammation and repair. Mammals have 3 TGF-β isoforms (1-3). During development, TGF-β3 is expressed at E13.5 in the connective tissue of the SS tendon, with a shift of expression to TGF-β1 at E15.5 (Table 1). No expression of either of the 2 isoforms is detected postnatally. The TGF-β protein is also found in pre-chondrogenic mesenchymal cells, but active TGF-β signaling ceases as cells differentiate into chondrocytes. TGF-β is also capable of inducing upregulation of both tenogenic genes (Sccx and tenomodulin) and chondrogenic genes (Sox9 and aggrecan), depending on the presence of transcriptional regulators and activation of intracellular signaling cascades. Akiyama et al showed that differentiation of pre-hypertrophic chondrocytes to hypertrophic chondrocytes is inhibited by Sox9 expression. Therefore, excessive expression of TGF-β may have a negative influence on development of the fibrocartilaginous zone. This implies that the microenvironment modulates TGF-β functions through strict control of transcriptional regulators (Fig. 2).

Altogether, this indicates an early regulation of enthesis development in which TGF-β directs the formation of the Sccx̂/Sox̂ progenitor pool. Tenocytes of the developing tendon then establish the formation of the attachment to the primary cartilage through Sccx/BMP-4 signaling to the eminence cells. Here, enthesis cell number expands, causing lengthening of the enthesis, followed by gradual cell differentiation postnatally, starting at the base of the enthesis and moving toward the tendon midsubstance. As the cells differentiate, they change collagen type expression, creating the extracellular matrix gradient. This differentiation of nonmineralizing fibrochondrocytes to mineralizing fibrochondrocytes is driven by Ihh expression. Together these steps lead to the formation of a mature enthesis with a cellular and mineralized gradient (Fig. 3).
The importance of biomechanical impact on the developing enthesis

Muscle loading of the developing enthesis is important for a functional mature enthesis. Lack of muscle load during development results in decreased enthesis mineralization, with disorganized cell patterns and lack of a fully developed 4-zone transition. Studies on paralyzed mouse shoulders demonstrated that mineralization occurred at a slower rate, with increased resorption of the mineralized fibrocartilage. The insertion of the enthesis into the humeral head on the paralyzed shoulder was found to be much more porous and to consist of nonmineralized cartilaginous tissue compared with the normal shoulder. The mineralization rate between the paralyzed and normal entheses was found to be unaffected, whereas the initiation of the mineralization front was delayed in the paralyzed shoulders. The researchers believed that the delay in mineralization initiation was due to muscle unloading affecting chondrocyte hypertrophy. Muscle unloading also increases the population of Ihh-responsive cells in late postnatal development, further supporting the role of muscle stimulation in enthesis maturation.

In regard to enthesis healing after RC repair, immediate and prolonged loading seems to provide adverse effects for enthesis healing. Furthermore, low-force loading improves healing, whereas complete removal of loading is deleterious. These results imply a delicate balance between improved or impaired healing and loading and require further data to provide firm recommendations for optimal postoperative loading for enhanced healing.

Inflammation of the enthesis

Studies of patients with RC tears have correlated an increase in tear size with a greater proinflammatory response in the adjacent synovium. This has been associated with the inflammatory degeneration of the enthesis after SS tears. As the enthesis is a site of high muscle loading and stress, it is subject to wear and tear with age, and therefore the inflammatory response at the synovium may be a secondary event, following release of damage-associated molecular patterns from entheseal damage. In full-thickness RC tears, increased numbers of inflammatory cells, such as myeloid cells, have been demonstrated in the inflamed synovial tissue. Upregulation of the proinflammatory mediators interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF), matrix metalloproteinase (MMP)-1, MMP-3, and MMP-13 has been demonstrated in the inflamed synovium and SS enthesis and in the subacromial bursa of RC tear patients. In addition, Gottoh et al observed degradation of the SS enthesis osteochondral tissue in torn RCs, where formation of granulation tissue with macrophages and multinucleated osteoclasts were found adjacent to the osteochondral tissue. These multinucleated cells are able to degrade bone tissue.

Figure 2  A schematic representation of the transforming growth factor β (TGF-β)–induced differentiation. TGF-β induces the specification of the multipotent Scx+/Sox9+ cell line from the progenitor cells of the primary cartilage, which forms the bone eminence from which the enthesis develops. These cells may differentiate to either tenocytes or chondrocytes by regulation of scleraxis (Scx) and SRY-box 9 (Sox9). After chondrogenesis, Indian hedgehog (Ihh) induces hypertrophic differentiation. At this stage, the chondrogenic cells downregulate Sox9 expression as it inhibits the transition from pre-hypertrophic to hypertrophic chondrocytes. Runt-related transcription factor 2 (Runx2) is expressed by pre-hypertrophic chondrocytes and initiates transcription of hypertrophic-related genes. Collagen type X (ColX) and alkaline phosphatase (AP) are expressed by the hypertrophic chondrocytes at the onset of mineralization. Tenocytes express the tenogenic transcription factor Scx. TGF-β also has the capacity to induce differentiation of mesodermal cells into both tenocytes and chondrocytes, depending on regulation of transcriptional regulators such as TGF-β–induced homeobox 1 (Tgif1).
multinucleated osteoclast cells is the receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL), which activate downstream transcription factors nuclear factor-κB (NF-κB), activator protein 1 (AP-1), and nuclear factor of activated T cells 1 (NFATc1), inducing osteoclastogenesis. The granulation tissue was found in almost all of the torn tendons, implying that the formation of granulation is a distinct entity in tendon degradation. Results from studies indicate that cartilage and proteoglycan degradation facilitates cellular invasion and formation of granulation tissue.

TNF is a major cytokine in the inflammatory response and induces expression of several other cytokines. IL-1β is secreted from macrophages and binds to IL-1 receptor 1 (IL-1R1) on target cells. IL-1β increases the production of MMPs and prostaglandin E2. The chemokine IL-8 is expressed by both synovial macrophages and synovial fibroblasts and serves to recruit monocytes and neutrophils to the inflamed synovium, contributing to osteoclast expansion and pannus formation. MMP-1, MMP-3, and MMP-13 are able to degrade proteins such as collagen types 1, 2, and 3 and proteoglycans and to regulate the activity of several cytokines.

IL-6 is an inflammatory cytokine with both proinflammatory and anti-inflammatory functions. IL-6 signal transduction through its membrane-bound receptor (IL-6R), classical signaling, is believed to mediate anti-inflammatory activities. Signal transduction through the soluble IL-6 receptor (sIL-6R), trans-signaling, is believed to mediate proinflammatory activities. On binding to its receptor, the IL-6/sIL-6R complex associates with the membrane-bound glycoprotein 130 (gp130), which then relays the signal. IL-6 attracts monocytes by inducing IL-8 synthesis from resident cells and induces monocyte differentiation into macrophages. IL-6, TNF, and IL-1 have also been shown to stimulate RANKL expression on synovial fibroblast and osteoblast cells, leading to osteoclastogenesis.

Although TNF and IL-1 depend on IL-6 for osteoclast-induced differentiation, studies have reported direct RANKL/RANK and IL-6-independent osteoclast differentiation by these cytokines. Bone marrow–derived macrophages (osteoclast precursor cells) can differentiate into osteoclasts directly after IL-1 stimulation without IL-6. Overexpression of IL-RI enables IL-1 to induce osteoclast differentiation without RANKL stimulation. Furthermore, RANKL-stimulated bone marrow–derived macrophages upregulate IL-1RI.
expression through the transcription factors c-Fos and NFATc1 while downregulating expression of the antagonistic receptor IL-RII. This indicates that IL-1 can induce osteoclast differentiation, after initial RANKL stimulation, thereby increasing bone erosion activity without the presence of IL-6.52 Another study found the combination of TNF and IL-6 to induce osteoclastogenesis in RANK-deficient mice.58 In this study, IL-6R was necessary for TNF/IL-6–induced osteoclastogenesis, demonstrating that the increase in the osteoclast population after TNF/IL-6 stimulation was largely due to increased cell proliferation.58 Histologic analysis showed indistinguishable cartilage damage between control and RANK-deficient mice.58 In addition, the study identified osteoclasts in the inflamed synovium of RANK-deficient mice. TNF signaling through TNF receptor 1 (TNF-R1) stimulates osteoclast differentiation by enhancing RANKL–induced osteoclast differentiation.52 TNF also stimulates osteoclast survival and inhibits the differentiation of osteoblast–precursor cells into mature osteoblasts.1,12 Together, this indicates several stimuli in the inflammatory environment for osteoclast differentiation, leading to excessive bone erosion.

IL-6 inhibits proliferation of synovial fibroblasts and stimulates expression of tissue inhibitor of MMP 1 (TIMP-1) in both synovial cells and chondrocytes.56,71 The inhibitory effect observed by IL-6 is suggested to be due to a negative feedback mechanism on TNF-stimulated proliferation of synovial fibroblasts, as TNF induces IL-6 production from these cells.56 Interestingly, though, both studies reported that the IL-6/IL-6R complex, and thus trans-signaling, mediated the inhibitory effect and production of TIMP-1, rather than the classical signaling pathway.56 Therefore, this may be a regulatory mechanism of the IL-6 proinflammatory response to control proliferation of synovial cells and MMP activity during elevated IL-6 levels. However, a study by Deon et al investigated the crosstalk between IL-6 and IL-1 in synovial fibroblasts.18 They found IL-1β to inhibit expression of IL-6–induced TIMP-1 and both IL-1β and TNF to modulate IL-6.18 Therefore, IL-6 may not inhibit the proliferation of synovial fibroblasts and MMP activity in the inflammatory environment. This suggests a complex interplay between the inflammatory mediators present, in which proinflammatory cytokines are able to regulate the activity of anti-inflammatory cytokines, thereby inducing chronic inflammation (Fig. 4). Altogether, these events tip the balance toward excessive degradation of the enthesis tissue matrix.

Administering anti-inflammatory drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), is a common treatment for reducing the inflammatory response after injury. However, a study by Cohen demonstrated that use of NSAIDs after acute SS repair in rats completely inhibited SS enthesis healing.13 The control group showed increased collagen organization over time, and fibrocartilage formation was noticed. However, the experimental group did not show any improvement over time and demonstrated a markedly disorganized collagen structure and no evidence of fibrocartilage formation. Osteoclast numbers decreased faster without NSAID treatment, and NSAID-treated rats revealed impaired osteoblast differentiation.13 These results indicate that NSAID treatment has adverse effects on enthesis healing, possibly by inhibiting osteoblast differentiation, and promotes further matrix degradation by excessive osteoclastic activity.13

MMP and TIMP matrix regulation

Turnover of the extracellular matrix is a dynamic balance between synthesis and degradation. This balance is essential for proper healing, leading to pathologic conditions when it is uncontrolled.13 MMPs are major mediators of remodeling, able to degrade essentially every component of the extracellular matrix.13 Activity of MMPs is regulated by their natural endogenous inhibitors, TIMPs. Balanced expression levels of MMPs and TIMPs are therefore critical in preserving the integrity of the extracellular matrix.

Modulation of MMP activity in the healing response also has the potential to improve clinical outcome. By inhibiting MMPs during healing, excessive degradation of the matrix and the severity of the degradative inflammatory response can be reduced.77 In a study using doxycycline to inhibit MMP activity in an acute rat SS injury model, both preoperative and postoperative administration demonstrated an increased development of fibrocartilage and significantly increased collagen organization in early healing compared with the control group.77 These results indicate that inhibition of MMPs during healing can potentially improve healing.77 In addition, a study by Gulotta et al found MMP-14, a membrane-bound MMP, to significantly improve enthesis healing after applying MMP-14–transduced mesenchymal stem cells (MSCs) compared with normal MSCs.31 Overexpression of MMP-14 increased fibrocartilage at the enthesis 4 weeks after unilateral detachment and repair of the rat SS tendon.31 Overexpression of MMP-14 at the enthesis also showed a significant increase in biomechanical strength with higher ultimate load and stress to failure and higher stiffness than in MSC-treated rats.31

SS enthesis healing

Unacceptable high retear rates after RC tendon repair are still reported.68 Improved suture techniques have not changed the numbers of tendon failures significantly. The major problem in the treatment of RC tears is the inadequate healing of torn tendons, as the enthesis does not regenerate its native structure. Instead, it heals in a scar-mediated fashion, leading to an enthesis with biomechanically inferior properties.14,24,37,41,44,50 The proliferation of injured enthesis cells and expression of collagen types 1 and 3 increase significantly after injury.24 In a rat SS tendon repair model, cell proliferation increased to a maximum 7 days after injury and thereafter decreased steadily over time but remained elevated compared with the preinjury level.24 Collagen type 1 and 3 mRNA expression reached a maximum at day 10, followed by a decrease to nearly normal levels 56 days after injury, but in contrast to type 1, the expression of type 3 remained elevated for several days longer before decreasing. Collagen type 1 accumulated slowly over time, whereas collagen type 3 followed the mRNA expression of collagen type 3.24 Normally, the enthesis does not contain significant amounts of collagen type 3. Therefore, elevated levels may indicate a remodeling response with immature, disorganized collagen type 3 in the early healing response, which is then subsequently replaced.24 This was supported by Kanazawa et al, who also investigated the structure of the healing enthesis at several time points after RC repair in rats.37 The study revealed low organization of collagen, with significantly reduced biomechanical properties. The collagen organization, mineralization, and biomechanical properties improved considerably from 4 to 12 weeks, although it never obtained properties comparable to those of the native enthesis.37 At 4 weeks, an increase in cellular density was observed, which decreased over time to normal, compared with the native enthesis. Interestingly, the study also revealed a change in the morphology of enthesis cells from a normal chondroid shape to an ellipsoidal shape. Likewise, cell processes also changed from oriented parallel to the adjacent collagen fibers to...
disoriented processes. These morphologic changes did not reverse to the native state. Several studies have found the expression of TGF-β to increase significantly after injury. Galatz et al found the expression of TGF-β1 to be localized at the site of scar tissue formation, whereas low TGF-β3 expression was confined to the uninjured tissue. The development of the collagen "patch" may therefore be due to increased TGF-β expression. This directs the deposition of collagen types 1 and 3 while also inhibiting chondrogenic differentiation, preventing the regeneration of the native enthesis.

There is mounting evidence that early intervention to restore the tendon-bone connectivity is an important strategy for maximizing recovery after RC tears. To prevent retear after RC repair, a number of studies have examined the effects of various biologic agents on tendon-to-bone healing in animal models. These techniques have included application of exogenous growth factors, growth factors in an engineered matrix, platelet-rich plasma, stem cell injections, and stem cells in an engineered matrix.
Several studies have investigated the potential of TGF-β3 to drive scar-mediated healing responses toward more regenerative healing responses.\textsuperscript{41, 44, 50} TGF-β3 induces chondrogenic differentiation of MSCs from torn RC tendons and reduces scar tissue formation in a cutaneous rat incision wound model.\textsuperscript{55, 68} By using a heparin/fibrin-based delivery system of TGF-β3, Manning et al reported improvements in SS enthesis healing after sustained delivery of TGF-β3 during a period of 6 weeks compared with control. Although TGF-β3 did lead to early improvements in biomechanical properties, these were still inferior to the uninjured enthesis.\textsuperscript{50} The treatment also did not lead to a reduction in scar tissue formation as expected, but instead a more disorganized scar tissue was noticed.\textsuperscript{50} This suggests that adult fibroblast tissue has a different response to TGF-β3 than progenitor cells present prenatally.\textsuperscript{50} A study by Kim et al found no improvements in biomechanical properties or reduction in scar tissue when delivering TGF-β3 and neutralizing antibodies for both TGF-β1 and TGF-β2 but did find indications of fibrocartilage at the repair site.\textsuperscript{41} The absence of improved healing may be due to the drug delivery method used, as antibodies were delivered to the bursal surface and not directly to the repair interface.\textsuperscript{41}

A study by Ousema et al investigated the effect of IL-1β on chondrogenesis of human MSCs.\textsuperscript{52} They found that IL-1 levels in diseased joints completely inhibited chondrogenesis. Even in treating with TGF-β3, chondrogenesis and biomechanical improvements were effectively inhibited.\textsuperscript{59} Wehling et al supported these results and found the same effect with TNF.\textsuperscript{58} Both IL-1β and TNF suppressed chondrogenic differentiation through NF-kB expression, and Sitcheran et al further found TNF to suppress chondrogenic stimulation through NF-kB by downregulation of Sox9 expression.\textsuperscript{72} Thus, using TGF-β3 alone might not be useful for clinical treatment.

Inhibition of TNF has also shown improved enthesis healing in rats at the early time points after injury.\textsuperscript{39} A study by Gulotta et al systematically inhibited TNF using the TNF inhibitor pegylated soluble TNF-R1 and found increased fibrocartilage and a significant improvement in load to failure and stiffness compared with untreated rats 4 weeks after SS repair.\textsuperscript{30} No differences in either cartilage formation or biomechanical strength were seen between groups 8 weeks after repair.\textsuperscript{30} Therefore, as most repair failures occur in the early stages of tissue healing, administration of cytokine inhibitors may enhance the strength of the early healing response, preventing repair failure. Data from a recent study by Honda et al suggest that clinical application of hyaluronic acid improves tendon-to-bone healing 4 and 8 weeks after RC repair.\textsuperscript{46} The study found hyaluronic acid to accelerate chondrogenic differentiation of MSCs and thereby increase the formation of fibrocartilage.\textsuperscript{38} Thus, future treatment plans may involve application of exogenous hyaluronic acid and MSCs to decrease the number of retears after surgery.

Collagen deposition, loss of a 4-zone structure, and absence of significant improvements in TGF-β treatment may reflect excessive TGF-β1 expression after enthesis tear. Different TGF-β genes signal through the same receptor complex. Therefore, released TGF-β1 may compete with TGF-β3 for receptor binding, leading to a reduction in the effectiveness of TGF-β3 treatment. However, cells in adult tissue may react differently to TGF-β3 stimulation than fetal cells. A possible treatment solution to improve healing could be a combination of TGF-β3 together with a cytokine antagonist and possibly TGF-β-neutralizing antibodies or MMP inhibitors.

Future discoveries may therefore present treatment with growth factors, cytokine inhibitors, scaffolds, and stem cell treatment to improve the development of a proper fibrocartilaginous transition and to increase the strength of the healing enthesis.

### Conclusion

In this review, we investigated existing knowledge about assembly, maturation, and degradation of the SS enthesis. Although recent discoveries have improved our understanding of the complex fibrocartilaginous entheses, much remains to be learned. More research into the inflammatory cascades taking place in the RC tissues after surgical repair will, it is hoped, pave the way for better clinical treatments.

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