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Original Research

Growth Factor Expression During Healing in 3 Distinct Tendons

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Purpose: We investigated unique tendon growth-factor expression profiles over time in response to simultaneous, similar injuries. Characterizing these genetic differences lays the foundation for creating targeted, tendon-specific therapies and provides insight into why current growth-factor treatments have success in some applications but not others.

Methods: The left fourth digital flexor, triceps, and supraspinatus tendons in 24 rats were cut to 50% of their transverse width at the midbelly under anesthesia. On postoperative days 1, 3, 5, 7, and 14, randomly selected rats were sacrificed, and the damaged tendons were excised and flash-frozen in liquid nitrogen. The expressional fibroblast growth factor 1, bone morphogenic protein 13, and transforming growth factor β-1 were measured at each time point and compared to their respective, uninjured levels with real-time polymerase chain reaction.

Results: The digital flexor tendon showed exponentially elevated expression of all 3 factors over the preinjury baseline values. Expression in the triceps and supraspinatus had more variation over time. The triceps tendon showed a considerable decrease of transforming growth factor β-1 and bone morphogenic protein 13 expression. The supraspinatus tendon had statistically significant increases of both transforming growth factor β-1 and bone morphogenic protein 13 expression relative to preoperative, uninjured levels, with a nonstatistically significant decrease of fibroblast growth factor 1.

Conclusions: Our study suggests different tendons express their own unique growth-factor profiles after similar, simultaneous injuries. The digital flexor showed particularly high, sustained levels of growth-factor expression in comparison to the supraspinatus and triceps, suggesting that variable dosing may be necessary for growth-factor therapies aimed at supplementing innate responses in these different tendon types.

Clinical relevance: These data show different tendons express unique trends of growth-factor expression over time in response to injury, suggesting each unique tendon may require specific dosing or knockdown therapies. These observations serve as a foundation for more tendon-specific questioning, experimentation, and therapeutic design.

Tendon injuries of the upper extremity and hand are exceptionally disabling to our patients. Much of this struggle is owed to the fact that tendon injuries are difficult to treat due to the limited ability of tendon to regenerate, as it is rarely able to remodel itself in a way that organizes collagen fibrils to preinjury strength and conformation.[1–4] Due to functional impairment, rehabilitation often takes months, with patients experiencing prolonged discomfort.

Efforts to improve clinical treatment of tendon injuries have traditionally focused on enhancing surgical techniques. However, recent research has shifted the focus from the biomechanical aspects of tendon repair to the biological processes underlying tendon healing itself. Genetic factors regulating growth factors coordinate the complex cascade of events required for ideal tendon healing, with over 400 recent studies dedicated to the topic.[5,6]

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However, it remains unclear whether different tendons display different levels of growth factors during healing in response to comparable injuries. This is clinically relevant, as current experimental and Food and Drug Administration–approved growth-factor therapies have shown limited success in a small subset of applications. These shortcomings are most likely due to a lack of understanding of how tendons in different locations and micro-environments differ in their native growth-factor requirements.[7,8]

Understanding the baseline growth-factor profile of nonrepaired tendon healing serves as a foundation for the development of therapies that may promote regenerative healing.[9,10] Growth factors, which are implicated in the scarless healing process and synthesized by fibroblasts, tenocytes, platelets, and inflammatory cells, serve to direct cellular mitogenesis and chemotaxis throughout the healing process of tendons.[11] Studies have demonstrated that injured extrasynovial tendons have higher degrees of adhesion formation, decreased strength, and decreased excursion as compared to intrasynovial tendons after surgical repair.[12,13] In contrast, intrasynovial tendons, such as the flexor tendons of the hand, have decreased glide resistance compared to other tendon types, with the vincula providing minimal blood supply. Therefore, nutrition is uniquely supplied mainly by the synovium and synovial fluid itself. Some extrasynovial tendons, such as the palmaris longus, receive microvascular supply from arteries supplying their associated muscles, while other extrasynovial tendons with a flatter morphology, such as the triceps and Achilles tendons, depend on musculotendinous junctions, osteotendinous junctions, and the paratenon for blood supply. Mixed tendons with both intrasynovial and extrasynovial segments, such as in the rotator cuff, receive blood supply from the arthrodial synovium in addition to musculotendinous and osteotendinous junctions.

We set out to characterize the growth-factor expression levels in 3 distinct tendons in response to an incision-based injury using microscissors in the rat. The growth-factor expression was analyzed at regular postincision time points in the left fourth digital flexor, triceps, and supraspinatus tendons. We measured expression levels of fibroblast growth factor (FGF) 1, bone morphogenic protein (BMP) 13, and transforming growth factor (TGF) β-1. These 3 growth factors were chosen due to their high frequency of study and characterization in multiple human and rat models of tendon disease.[14–16] Given the variations in blood supply to each tendon type, the healing cascade of growth factors required to draw in the appropriate inflammatory cellular response is likely different as well.[12,17] As such, we hypothesized that each of the 3 tendons tested would express their own unique growth-factor profile after similar, simultaneous injuries. The purpose of the study was to assess differences in growth-factor expression within and between tendons to support the design and creation of therapeutics that promote tendon-specific healing regimens and expedite return to function. Our hypothesis is that postoperative relative expression levels of all 3 growth factors vary significantly from their baseline uninjured values.

Materials and Methods

Rat model preparation

All animal surgeries were performed at the University of Chicago's Animal Resource Center to maintain a controlled environment. The Sprague-Daley rat species was chosen for our study due to genetic homogeneity and wide availability. Each rat was anesthetized with isoflurane delivered through the chamber before transition to a nose cone in a lateral decubitus position on the operating table. After induction, the shoulder and right arm were shaved, and the skin was prepped and draped in a sterile manner. All lacerations were made with microscissors by the same surgeon.

Rat tendon transections

An incision was made over the palmar aspects of the proximal phalanges of the second through fifth toes of the right forelimb limb. The digits were extended, and a 50% laceration was created in the deep flexor tendon just proximal to the A1 pulley of the third digit, leaving the superficial flexor tendon and the tendon sheath intact. The skin was closed with nylon sutures for all 3 tendons. For the rotator cuff tendon, a 2-cm incision was made over the dorsal aspect of the shoulder. The scapular spine was identified, and a portion of the trapezius and deltoid was released to allow exposure of the rotator cuff. After direct visualization of the supraspinatus muscle, a 50% laceration was made to the tendon just distal to the musculotendinous border. The deltoid was reapproximated over the rotator cuff before closure. The triceps tendon was approached via a direct posterior approach with an incision over the skin of the right forelimb directly proximal to the elbow joint. After incising the peritenon in line with the skin, the triceps tendons were isolated. A 50% laceration was made to the triceps tendon at a point halfway between the musculotendinous junction and the insertion onto the olecranon (Fig 1). Immobilization was not required because half of each lacerated tendon remained intact, allowing for safe use and the most humane treatment of our subjects.

Rat tendon specimen collection

The rats were allowed to move about freely in their cages with continued access to food and water. Postoperative analgesia was provided in the form of 48 hours of intramuscular meloxicam. There were 6 groups representing each time point, with 4 rats in each group, for a total of 24 rats. The same injury was delivered on the right side of all rats. Randomly selected rats were sacrificed at each postoperative day (1, 3, 5, 7, and 14). Prior to euthanasia, a second round of anesthesia was induced with isoflurane for live excision of tissue. A wide excision of the injured flexor tendon, rotator cuff, and triceps tendon was resected en bloc and immediately snap-frozen in liquid nitrogen and stored at −80 °C. On the day of tissue processing, each en bloc excision was trimmed to 6-mm proximal and distal to the artificial wound bed prior to homogenization in liquid TRIzol reagent (Thermo Fisher) and RNA extraction. Unfortunately, during recovery, 3 rats developed surgical site infections, which were treated with antibiotics and local wound care. However, these rats were not included in our study; therefore, our final study data include 21 rats. Analyses for baseline day 0 and postoperative days 1 and 3 included 4 rats, while postoperative days 5, 7, and 14 included 3 rats.

RNA isolation, reverse transcription, and quantitative reverse transcription–polymerase chain reaction

Frozen tendon samples were cut into small pieces and then added to 1 mL of TRIzol reagent. After 5 minutes of homogenization, the lysate was transferred to a 2-mL RNase-free Eppendorf tube and kept on ice. Next, 270 μL of chloroform was added to each tube, followed by vortexing. The samples were held at 4 °C and centrifuged at 12,000 g for 15 minutes. The upper aqueous phase was transferred to fresh 1.5-mL Eppendorf tubes and kept on ice. Next, 800 μL of isopropanol was added to precipitate the RNA and the mixture was centrifuged at 12,000 g for 10 minutes at 4 °C. After removal of the supernatant, RNA pellets were washed with 600 μL of 75% ethanol twice and dissolved in 50 μl of RNase-free water. Two μL of Hexamer (0.5 μg/μL) combined with 10 μL

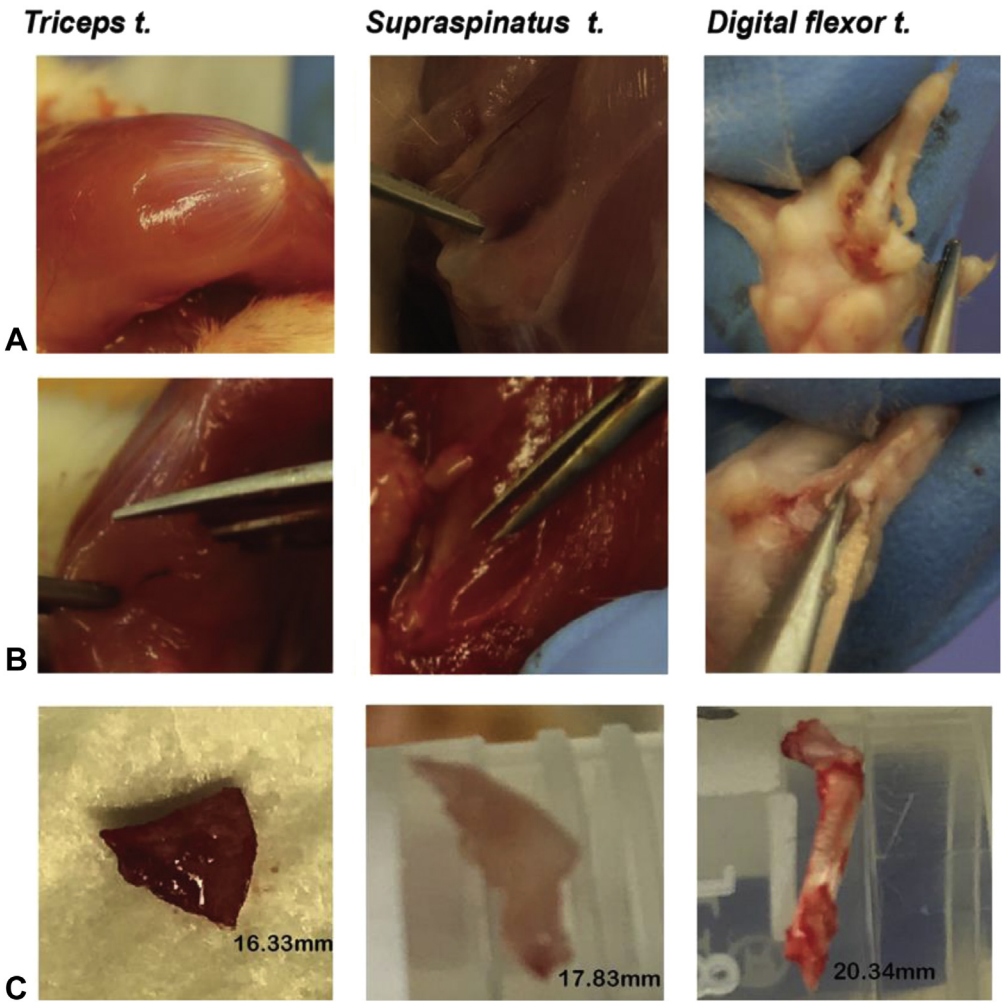

Figure 1. Simultaneous tendon injury in 3 distinct types. **A** In situ photographs of the 3 upper extremity tendons chosen for experimental injuries: triceps, supraspinatus, and the fourth digital flexor, from left to right. These tendons were selected because each exists within a very different microenvironment. **B** Representative photos of our 50% tenolysis injury model using microscissors. **C** Photos of each tendon type after harvest, just before flash freezing and storage at −80 °C. The length of each en bloc resection is depicted in millimeters. Messenger RNA was isolated at a later date after using phenol-based homogenization.

(approximately 10 pg) of total RNA was incubated at 70 °C for 5 minutes. A reverse transcription mix (Thermo Fisher) was made by combining 5 μL of 5X first-strand buffer, 2 μL of 0.1-M dithiothreitol, 1 μL of 10-mm deoxynucleotide triphosphates, and 0.2 μL of RNAsin. The Hexamer-RNA mix was combined with 6.5 μL of the reverse transcription mix and 1.5 μL of reverse transcription enzyme and incubated for 60 minutes at 37 °C, followed by 1-minute incubation at 95 °C. Using double distilled water, complimentary DNA was diluted to 1:100 stocks.

Real-time polymerase chain reaction analysis was performed using DNA Engine Opticon (MJ Research Inc). Primers for each growth factor and glyceraldehyde-3-phosphate dehydrogenase control are listed in the Table. The polymerase chain reaction mixtures contained 5 μL of 2X Dynamo (Cat#: 145, Finnzymes, MJ Research Inc), 1.25 μL each of growth-factor specific forward and reverse sequence primers (40 ng/μL), and 2.5 μL of the cDNA template. The conditions used for polymerase chain reaction were an initial 2 minutes at 95 °C, followed by 35 cycles of 94 °C for 20 seconds, 56 °C for 20 seconds, 72 °C for 20 seconds, and a primer-specific temperature for 1 second, during which the fluorescence measurement was made. All results were interpreted by standard curve comparison and normalized to glyceraldehyde-3-phosphate dehydrogenase as the control. Relative expression levels at different time points were compared to uninjured baseline levels using a moderated Student *t* test with Bonferroni correction for multiplicity. The mean and standard error of the mean are reported. The Student *t* test was chosen to compare baseline expression to postoperative values, as this this was best fit to assess our working hypothesis that postoperative values were significantly different than uninjured, baseline values.

Table
Polymerase Chain Reaction Primer Sequences for Measured Growth Factors and GAPDH*

Growth Factor	Forward Primer (5′-3′)	Reverse Primer (3′-5′)
TGFβ-1	GCAGTGGCTGAACCAAGG	GGTCACCTCGACGTTTGG
FGF-1	TGGGCCTCAAGAAGAACG	GGGAGCACCCAGAACAGA
VEGF	CCTGTGTGCCCCTAATGC	TGCTGGCTTTGGTGAGGT
BMP-12	GCTGGGACGACTGGATCA	TGAGCAGCGTCTGGATGA
BMP-13	AAGCGACACGGCAAGAAG	CGCAGTGATAGGCCTCGT
GAPDH	TGGATGGTCCCTCTGGAA	GTGAGCTTCCCGTTCAGC

* The Table represents the genetic sequences used for each reverse transcription polymerase chain reaction primer in our investigation. Care was taken to use species-specific primers with validation in commercial testing or previous studies. Glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene and delta-delta cycle threshold methods implemented to determine relative expression levels of our growth factors of interest. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor.

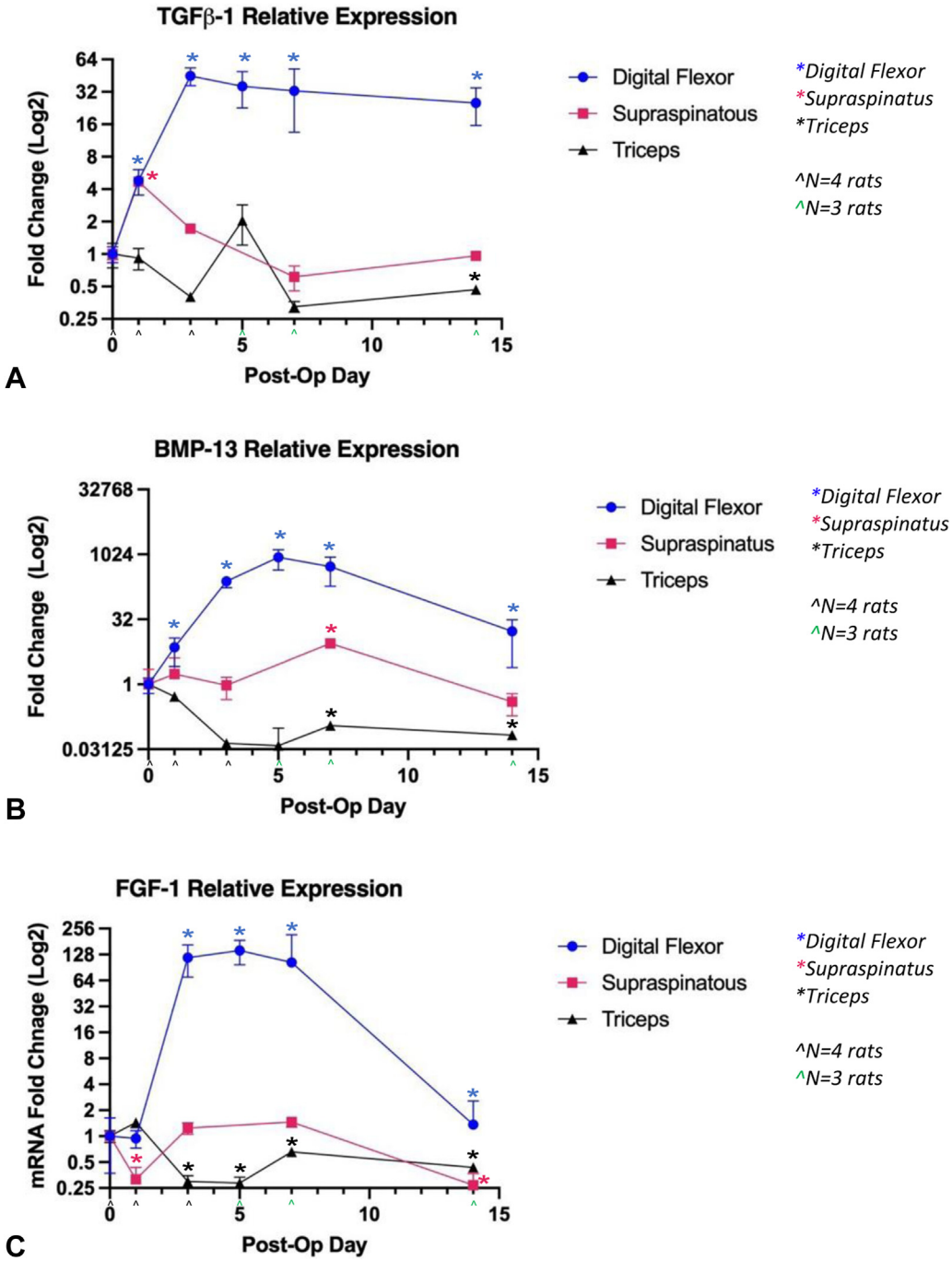

Figure 2. Growth factors TGFβ-1, BMP-13, and FGF-1 show different expression patterns over time following injury in different tendon types. **A**–**C** Plots of the mean relative expression levels of TGFβ-1, BMB-13, and FGF-1, respectively. Day 0 represents tissue expression in biologic replicates that did not undergo surgery. Day 0 serves as our baseline for postoperative expressional changes. Baseline relative expression of each growth factor was compared to postoperative dates within the same growth factor and same limb only. Hypothesis testing using a Student *t* test with Bonferroni correction for multiplicity was used to test each postoperative time point against a single baseline time point. *A time point for each growth factor that is significantly different than that same growth factor at baseline, with an uninjured day 0 expression *P* value <.05. Error bars represent the standard error of the mean (n = 21). ^There were 3 or 4 rats for each postoperative day. mRNA, messenger RNA.

Results

The digital flexor tendon showed the highest relative expression of all 3 growth factors

The digital flexor tendon was unique in that it was observed to have rapid and sustained upregulation of all 3 growth factors in response to a 50% transectional injury (Fig 2). The digital flexor tendon was found to have the highest relative peak expression of all 3 growth factors at any given time point compared to the triceps and supraspinatus. All postoperative elevations of TGFβ-1, FGF-1, and BMP-13 were significantly higher than their respective baselines, except for day 1 in FGF-1 ($P < .05$ at each time point). When TGFβ-1, FGF-1, and BMP-13 levels were compared over time within the digital flexor itself, BMP-13 had the highest relative increase over day 0, uninjured levels compared to TGFβ-1 and FGF-1 at every

time point except for day 14 (Fig 2). Bone morphogenic protein 13 had a maximum fold change of 875 over baseline at postoperative day 5 (Fig 2B). At day 14, both BMP-13 and TGFβ-1 were found to be significantly elevated to more than 20-fold of preoperative, day 0 values in the digital flexor tendon ($P < .003$ and $P < .02$, respectively; Fig 2A and B).

The triceps tendon consistently showed general decreases of TGFβ-1, FGF-1, and BMP-13 in response to injury

The triceps tendon, a flat tendon with no synovium, was interestingly the only tendon that showed consistent decreases in expression of all 3 growth factors in response to injury, especially for BMP-13 and FGF-1 (Fig 2). Bone morphogenic protein 13 expression was found to be significantly lower than baseline at each time point ($P < .01$; Fig 2B). Fibroblast growth factor 1 expression was also found to be significantly lower at each time point, except for postoperative day 1, when levels were similar to those found before surgery ($P < .03$), Fig 2C). Transforming growth factor β 1 expression was decreased as well in response to injury in the triceps, but was only found to be statistically decreased at postoperative days 3, 7, and 14 (Fig 2A).

The supraspinatus tendon had statistically significant increases of both TGFβ-1 and BMP-13 expression, with a nonsignificant decrease of FGF-1 over time

The supraspinatus showed the least variance from baseline of all 3 growth factors in response to injury, with very few time points being significantly different from baseline ($P < .05$, Fig 2). For TGFβ-1, only postoperative day 1 was significantly different from baseline, with a 2-fold increase in expression ($P = .032$, Fig 2A). Similarly, for BMP-13, only day 7 was found to be statistically different from baseline, with a mean increase of 28.2-fold over baseline ($P = .014$, Fig 2B).

Discussion

In this study, we measured growth-factor expression in response to injuries between 3 different tendons; 1 intrasynovial, 1 extrasynovial, and 1 mixed tendon type spanning both intrsynovial and extrasynovial regions. One of the key findings of our study was the observation that the intrasynovial digital flexor tendon had markedly elevated and sustained expression of all 3 growth factors studied: TGFβ-1, FGF-1 and BMP-13. In fact, expressional levels of TGFβ-1, FGF-1, and BMP-13 were highest in relative measures at each time point for the digital flexor when compared to the supraspinatus and triceps tendons.

An improved understanding of the differences in growth-factor expression during tendon healing lays a foundation to create novel therapies to improve outcomes following tendon injury. Furthermore, treatments could be specifically designed based on the type of tendon being repaired and the type of tendon autograft used. Kashiwagi et al[18] demonstrated that the addition of TGFβ to injured rat Achilles tendons improved tensile strength via an increase of type I collagen deposition and physiologic orientation. A comparable study performed using several isoforms of BMP garnered similar results.[19] Bone morphogenetic protein, also referred to as cartilage-derived morphogenetic protein, was initially implicated in bone formation, but it is now known to have extensive tendon-forming activity as well.[20] In addition, Costa et al[21] noted that the combined application of insulin-like growth factor 1, platelet-derived growth factor (PDGF), and FGF to rabbit flexor tendons exhibited a synergistic increase in tenocyte proliferation. Separately, platelet-derived growth factor and FGF have been shown to increase the expression of type 1 collagen when added to intrasynovial flexor tendons.[22,23] Vascular endothelial growth factor has also been shown to be a powerful angiogenic stimulator, promoting vascular ingrowth to the site of the tendon injury.[24] All of these previously mentioned growth factors, except for BMP, have been shown to be upregulated in injured tendons.[25–28]

Digital flexor injuries are challenging to treat, with previous studies demonstrating mixed results of growth-factor therapy applied during repair in the rat.[11] Our data suggest that the natural healing cascade of the digital flexor tendon requires higher levels than baseline when compared to our other tendons tested. Whether this trend is an innate quality of all intrasynovial tendons or just the digital flexor remains unknown and provides yet another avenue for future studies.

Another exciting finding is that the triceps tendon demonstrated decreased expression of all 3 growth factors—TGFβ-1, FGF-1, and BMP-13—in response to an injury. Specifically, FGF-1 and BMP-13 showed consistent reductions in response to injury at almost every time point over baseline, with the exception of postoperative day 1 for FGF-1. Transforming growth factor β-1 was observed to be initially suppressed over the first 3 days, with a precipitous rise to 2 times the baseline expression at day 5, before returning to baseline values at day 14. The triceps tendon is rarely studied as an injury model, and these data suggest that the initial inflammatory phase of healing does not universally involve the upregulation of TGFβ-1. In contrast, these data are very different than previous injury models in the rat Achilles tendon, which implicated increased levels of TGFβ-1 expression throughout the healing period.[29,30]

The Achilles is by far the most common flat tendon studied in rodent and human models, with studies almost universally implicating TGFβ-1 expression in the early healing period.[5,9,18,29,31] Applied in this context, our data suggest that growth-factor expression may differ greatly between similar tendons at different locations and under different mechanical stresses. Indeed, there are many external factors besides tendon type and location that could contribute to variations in healing and growth-factor expression, including the mechanical load, local environment, and ability to control the degree of the postoperative activity level.[32–35] We tried our best to control for the injury type and location by cutting transversely to 50% width at the midbelly of the musculotendinous junction, with the excpetion of the digital flexor, which was cut at a point mid-digit. It is possible that each injury location has a different microenvironment of its own, and that expression would vary within the same tendon itself if injured and harvested at different locations along the same tendon. These questions are ones we would like to address with future study. It may be that every tendon has its own unique growth-factor "fingerprint," driven by its ireproducible mechanical and physiologic microenvironment, or it may be that similar tendons can be grouped according to similar genetic responses to injury. Future studies could begin investigating this hypothesis by repeating a similar study to ours but with injuries to multiple sites within the same tendon, in addition to different tendons that lie in closer proximity to each other.

One aspect of wound healing we mentioned, but did not study, is the level of native growth-factor protein embedded in each tendon type before the injury. As mentioned, latent extracellular-matrix embedded growth factors are released in response to tissue damage, serving as important initial chemotractactants. The baseline level of embedded growth factor in each tendon type compared to the other is unknown. Additionally, correlation of gene expression levels of TGFβ-1, FGF-1, and BMP-13 to histologic and biomechanical features of optimal healing at each time point would be a logical next step to best identify the ideal growth-factor milieu for each healing environment and correlate growth-factor expression to a true functional phenotype.

Further limitations of this study include the possibility of specimen contamination upon resection during rat tendon specimen collection. Other tissues and associated contamination may have altered subsequently measured growth expression levels, especially given the small size of the resected tendons. Nevertheless, our surgery team conducted each transection and resection in a similar fashion with respect to the same anatomic landmarks in each rat, minimizing the potential error introduced by specimen contamination.

In conclusion, our study compares side-by-side healing between tendons on a genetic level. We were able to observe 2 key findings during our work: (1) the digital flexor tendon has a more robust growth-factor response relative to uninjured baseline expression than the supraspinatus and triceps when compared to their respective day 0, uninjured levels; and (2) the triceps tendon showed downregulation of TGFβ-1 during the initial healing period, inconsistent with results of many previous and comprable studies. These data combined suggest that different tendons indeed have their own unique growth-factor trends over time, some requiring more and some requiring less than prior to injury levels. The development of tendon-specific growth-factor combinations and dosing to gain the maximum clinical benefit is likely critical in harnessing the maximum benefit.

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