Published in final edited form as:

Connect Tissue Res. 2013 ; 54(3): 218–226. doi:10.3109/03008207.2013.787418.

Cellular and Molecular Factors in Flexor Tendon Repair and Adhesions: A Histological and Gene Expression Analysis

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Abstract

Flexor tendon healing is mediated by cell proliferation, migration, and ECM synthesis that contribute to the formation of scar tissue and adhesion. The biological mechanisms of flexor tendon adhesion formation has been linked to TGF- β . To elucidate the cellular and molecular events in this pathology, we implanted live FDL grafts from the reporter mouse *Rosa26^{lacZ/+}* in WT recipients, and used histological β -galactosidase (β -gal) staining to evaluate the intrinsic versus extrinsic cellular origins of scar, and RT-PCR to measure gene expression of TGF- β and its receptors, extracellular matrix (ECM) proteins, and MMPs and their regulators. Over the course of healing, graft cellularity and β -gal activity progressively increased, and β -gal-positive cells migrated out of the *Rosa26^{lacZ/+}* graft. In addition, there was evidence of influx of host cells (β -gal-negative) into the gliding space and the graft, suggesting that both graft and host cells contribute to adhesions. Interestingly, we observed a biphasic pattern in which *Tgfb1* expression was highest in the early phases of healing and gradually decreased thereafter, whereas *Tgfb3* increased and remained upregulated later. The expression of TGF- β receptors was also upregulated throughout the healing phases. In addition, type III collagen and fibronectin were upregulated during the proliferative phase of healing, confirming that murine flexor tendon heals by scar tissue. Furthermore, gene expression of MMPs showed a differential pattern in which inflammatory MMPs were highest early and matrix MMPs increased over time. These findings offer important insights into the complex cellular and molecular factors during flexor tendon healing.

Keywords

Flexor tendon; Tendoplasty; Autograft; Allograft; Adhesions; Tenocytes; Transforming Growth Factor; Extracellular Matrix; Matrix Metalloproteinase

INTRODUCTION

Each year, more than 4.8 million emergency department (ED) visits, or about 4% of the total ED cases, involve acute injuries to the fingers or hands [1] resulting in significant loss of

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DECLARATION OF INTEREST

The Authors have no conflict of interest to declare.

work days, second only to back pain and sprain [2]. Common among these are flexor tendon injuries [3]. The most persistent problem in these injuries is the formation of adhesions that lead to compromised joint flexion or joint contracture. The rate of these complications can be as high as 30–40% of the repaired or unrepaired cases [4]. Clinical and experimental observations suggest that formation of adhesions is precipitated by injury to the tendon sheath and inflammation, surgical manipulation, and immobilization [5], especially in injuries involving Zone II, which was once known as Bunnell's *no man's land* [6].

As an alternative to primary repair, flexor tendoplasty allows the surgeon to place the autograft or allograft ends outside of the confines of the flexor sheath in zone II, where they can be attached to the injured flexor tendon distally in Zone I and proximally in Zone III. Flexor tendoplasty can be the only option in cases of delayed primary repair due to infection or other complications [7]. However, debilitating adhesions have also been reported in 30–40% of clinical cases of flexor tendoplasty procedures involving live autografts [4], which underscores the need to identify the etiology of adhesions in order to develop effective tissue-engineered based alternatives to autografts. To date, this problem remains poorly understood [8], and no effective treatments have provided satisfactory outcomes, except for rehabilitation treatments using early controlled passive motion regimens [9, 10], which frequently still fail.

Animal studies suggest that tendon autografts mount an intrinsic response to the injury in both the tendon stumps and the live autograft itself that leads to adhesions [11–13]. For example, it was observed in the chicken model, that early repair of the FDP tendon-graft is mediated by proliferation and ingrowth of the epitenon cells and newly-formed collagen fibers indirectly suggesting that the live tendon autograft plays an active role in the repair [14]. Canine models, which represent the standard model in this field, have demonstrated that the source of the donor autograft (intrasynovial versus extrasynovial) is an important determinant of the fate of repair [12, 13, 15]. For example, extrasynovial tendon autografts have been shown to undergo extensive cellular death and rapid remodeling associated with the ingrowth of fibrovascular scar from peripheral tissue that reduces tendon gliding, while intrasynovial autografts experience minimal cell necrosis and matrix turnover [12, 13, 15], and result in decreased friction during gliding compared to the extrasynovial grafts [12, 13, 15]. However, despite the extensive clinical and preclinical literature on flexor tendon adhesions, there remains a paucity of definitive data about the relative roles of the live graft and host cells and the molecular signals in scar formation.

In the current study, we set out to determine the role of the live graft tenocytes and molecular signals in adhesions associated with surgery in a previously developed mouse model of flexor digitorum longus (FDL) tendoplasty [16]. In this model, we previously observed remarkable histologic differences between live autograft and acellular allograft healing that were manifested by the amount of fibrotic scar tissue surrounding the grafts [16]. While autografts were encased by scar tissue that appeared to be invading the tendon, the freeze-dried allografts experienced less scarring and remained mostly acellular at 14 and 28 days post grafting. These distinct modes of repair were consistent with the observed increased biomechanical impairment in the metatarsophalangeal (MTP) joint flexion in the autografts at 28 days [16]. In parallel studies, we demonstrated that early expression of *Tgfb1* in FDL tendon repair was associated with suppression of early expression of *Mmp9* and scar formation, while late expression of *Gdf5* was associated with upregulation of *Mmp2* and *Mmp14*, and improved MTP joint flexion thereafter [17]. We further confirmed the pro-scarring role of TGF- β by demonstrating that TGF- β /Smad3 loss-of-function in *Smad3*^{-/-} mice leads to improved FDL tendon gliding and MTP joint flexion following surgical repair [18].

The mouse model is not without limitations, the most important of which is the inability to induce injury and repair in zone II and the stress shielding of the repair site. While there could be concern about the translational relevance of mouse models, signaling pathways and disease processes are highly conserved across mammalian species, and studies in mice have provided insights about disease and have led to important new therapies in areas including cancers, heart disease, hypertension, diabetes, obesity, osteoporosis, glaucoma, blindness and deafness, and neuropathologies [19], and various skeletal tissue repair processes including tendon repair [20]. Therefore, we believe that the advantages of the mouse model outweigh its limitations.

Therefore, we hypothesized that tenocytes in the live graft contribute significantly to the formation of scar tissue in the vicinity of the graft, in association with TGF- β 1 signals that induce a biphasic regulation in MMP expression. To test this hypothesis, we transplanted live FDL tendon grafts from *Rosa26^{LacZ/+}* donor mice into intercalary FDL tendon defects of WT littermates. The repairs were assessed over time histologically using β -galactosidase (β -gal) staining, and gene expression of TGF- β and its receptors, extracellular matrix (ECM) proteins, and MMPs and their regulators were quantitatively measured using real-time RT-PCR.

MATERIALS and METHODS

Mouse Breeding

University of Rochester animal care committee approved all the protocols. *Rosa26^{LacZ/+}* [(B6.129S7-Gt(ROSA)/26Sor/J)] mice (Jackson Lab, Bar Harbor, ME), which carry retroviral insertion of bacterial *lacZ* (gene for β -gal) in one allele, were maintained in C57Bl/6 background. The colony was expanded by cross breeding *Rosa26^{LacZ/+}* Het and WT pairs. Genotyping was done by PCR. *Rosa26^{LacZ/+}* mice served as FDL tendon live graft donor and wild type (WT) littermates served as recipient. Mice were entered in the study at 3 months of age. The experiment was divided into 2 groups: (i) histological and (ii) mRNA expression studies.

Surgical Procedures

At each time-point, 3 FDL tendoplasty surgeries were performed for histological analysis and 6 were performed for RNA expression analysis. Mice were anesthetized using intraperitoneal injections of ketamine (60 mg/kg body weight) and xylazine (4mg/kg body weight). Surgeries were performed with the aid of 5 $\times$ magnification loupes against microsurgical background material (Accurate Scientific and Surgical Instruments). Briefly, a longitudinal plantar incision was created on the hindfoot of a WT mouse. A 3-mm defect was created in the FDL tendon, which was then reconstructed by transplanting a live FDL graft from an age matched *Rosa26^{LacZ/+}* donor using a 9-0 Ethilon suture (monofilament nylon, tapercut V100-3, Ethicon), as previously described [16]. The tendon was then transected at the proximal musculotendinous junction to temporarily immobilize the flexor mechanism to protect against disruption of the tendon graft early during the repair period and to eliminate early tendon gliding to induce adhesion formation [16]. The skin was closed using 5-0 Ethilon nylon suture (668G, Ethicon). Mice were sacrificed at different time-points between days 3 and 35 post-surgery, and the reconstructed tendons were harvested for analysis. The unoperated FDL tendon tissue was also recovered from WT mice for histological comparison. The mice were allowed free, unrestricted age activity upon recovery from anesthesia.

Histology, X-Gal, and Hematoxylin and Eosin (H&E) Staining

Reconstructed tendons were dissected with the surrounding soft tissue. The tissue was fixed in 3-ml freshly diluted cold 4% PFA (methanol-free, formaldehyde, EM Sciences) for 1h, washed with cold PBS three times 10 minutes each, transferred to cold 15% sucrose solution (in PBS) with gentle rocking for 3 hours followed by transfer to 30% sucrose for overnight in a cold room at 5°C with gentle rocking. The tissue was rinsed three times 5 minutes each in OCT compound (Tissue-Tek) and placed in a cryomold (Tissue-Tek) filled with OCT. Sections (7µm thick) were cut using a Cryostat (Thermo) and Accu-Edge blades (SAKURA).

Frozen sections were stained for β-gal using X-Gal as a substrate (Invitrogen). Briefly, frozen slides were warmed to 4°C, fixed in cold freshly diluted (in PBS) 0.2% glutaraldehyde (EM Sciences) for 10 min, washed once in PBS, once in wash solution (PBS containing 2mM MgCl₂ and 0.02% NP-40), and once in staining solution [wash solution containing 5mM K₄Fe(CN)₆ and 5mM K₃Fe(CN)₆], for 5 min each. Finally the slides were placed in a Couplin jar containing 'working solution' of X-gal (bromo-chloro-indolyl-galactopyranoside) substrate at 1mg/ml concentration. The slides were placed at 35°C for 8 hours for color development. The slides were then rinsed twice in PBS for 15 seconds each and counter stained with nuclear fast red (EM sciences) for 10 minute. After staining, the slides were rinsed in PBS and were then dehydrated in a gradient of ethanol (70%, 95%, 100%), 5 min each, twice. The sections were cleared by passing through Xylene, twice, 5 min each and were mounted permanently with Cytoseal 60 (Thermo) mounting medium and cover slipped. Frozen sections of unoperated FDL tendons were processed similarly and stained with H&E (Sigma Chemicals) using standard protocols.

RNA Extraction and Real-Time RT-PCR

For total RNA isolation, the reconstructed tendon including the graft and about 1.5 mm of the host FDL tendon proximal and distal to the graft-tendon junctions as well as surrounding scar tissue were carefully dissected from 6 animals at each time point, and were placed in sterile tubes containing 20 ml RNeasy lysis solution (Qiagen) at room temperature. The tissue pieces were transferred into Trizol reagent (Invitrogen) in which they were homogenized and total RNA was isolated using standard protocols. cDNA was prepared from 1µg total RNA in a 20µl reaction mixture in a 0.2ml tubes (Biorad) using M-MLV Reverse Transcriptase system (Invitrogen) following the manufacturer's protocol. Triplicate measurements from each cDNA sample were performed using SYBR Green (Applied Biosystems) according to the manufacturer's protocol. Briefly, a 20µl reaction mixture contained 0.5µl cDNA, SYBR Green and 0.5µM of the forward and reverse primer for mouse gene. The expression of target genes was measured using the Rotor-Gene Q (Qiagen) RT-PCR systems. Unless the source is acknowledged, the primer sequences were designed using Primer3 software and validated with NCBI's Primer-BLAST for specificity to the desired genes (Table 1). Due to technical difficulties that led to extremely low yield and quality of the mRNA extracted from normal tendons or day 0 repairs, gene expression was normalized with the housekeeping gene *Actb* (β-actin), and further normalized to the day-3 transcript levels.

Statistical Analysis

Data were analyzed using a one-way ANOVA to assess differences in gene expression levels over time followed by Tukey's multiple comparisons ($\alpha=0.05$).

RESULTS

Graft Cells Contribute to the Formation of Adhesions

Histologic sections revealed important cellular aspects of the *Rosa26^{LacZ/+}* graft healing response following transplantation. Wherein the unoperated control FDL tendon sections featured the characteristically organized fibrous matrix with sparse cellularity and a distinct gliding space between the epitenon and the fibro-osseous sheath and plantar fascia (not shown), the implanted grafts (Fig. 1) showed progressive cellular proliferation and migration leading to adhesions and loss of gliding space, and prolonged remodeling of the graft matrix. On day 7 (Fig. 1A–D), there was evidence of an influx of host cells (β -gal-negative) into the granulation tissue that invaded the gliding space, which was concomitant with a modest outward flux of β -gal-positive cells (Fig. 1D). On day 14 (Fig. 1E–H), there was a significant increase in graft cellularity and β -gal activity, and more pronounced outward flux of β -gal-positive cells from the graft, which appear to be contributing to the adhesions that obliterated the gliding space (Fig. 1G, H). On day 21 (Fig. 1I–L), there was a noticeable increase in β -gal-positive cells in the tendon graft and surrounding adhesions, and the graft margins were not distinguishable from the surrounding tissues (Fig. 1K, L). There was also a noticeable disorganization of the graft and a loss of the characteristically crimped fibers on days 21 and 28 (Fig. 1M–P), with no clear margins separating the graft from the surrounding adhesions.

Transforming Growth Factor Gene Expression

Tgfb1 (transforming growth factor β 1) mRNA expression was highest on day-3 (Fig. 2A) and decreased significantly over time reaching a minimum of 62% of baseline on day 28. In contrast, *Tgfb3* (transforming growth factor β 3) mRNA expression increased from baseline ~5-fold on day 14, and remained significantly ($p < 0.05$) upregulated thereafter (Fig. 2A). The expression of TGF- β receptor *Tgfr2* (transforming growth factor, beta receptor II) was increased on day 7 and remained elevated throughout the healing time course (Fig. 2B). *Acvr11* (activin A receptor, type II-like I or *Alk-1*) and *Tgfr1* (transforming growth factor, beta type 1 receptor or *Alk-5*) showed bimodal temporal changes in which they significantly ($p < 0.05$) increased on day 7, but decreased to baseline thereafter until they increased significantly ($p < 0.05$) again on day 35 (Fig. 2B).

Extracellular Matrix (ECM) Gene Expression

The expression of the fibrous ECM proteins (collagens I and III and their crosslinking enzyme lysyl-oxidase) and non-fibrous ECM proteoglycans, (biglycan, decorin, and versican) was assessed with RT-PCR (Fig. 3). *Col1a1* (collagen, type I, alpha 1) mRNA expression significantly ($p < 0.05$) increased up to day 14 by about 5-folds compared to the expression on day 3 ($p < 0.05$), and decreased thereafter (Fig. 3A). Expression of *Col3a1* (collagen, type III, alpha 1) mRNA showed a steeper significant increase ($p < 0.05$) up to 21 days to nearly 30-folds the expression on day 3 ($p < 0.05$), and decreased thereafter (Fig. 3A). *Lox* (lysyl oxidase) mRNA expression in general followed the expression profiles of collagen, peaking on day 14 and decreasing thereafter (Fig. 3A). We also examined the expression of genes that mediate cell-ECM interaction and adhesions using RT-PCR. *Acta2* (smooth muscle actin, alpha 2 or α -SMA) mRNA expression increased significantly by 4 folds ($p < 0.05$) on day 7, and decreased thereafter but remained elevated compared to day 3 (Fig. 3B). *Fnl1* (fibronectin) mRNA expression increased progressively and significantly over time, by up to 6-fold on day 35 compared to day 3 (Fig. 3B).

Matrix Remodeling (MMPs and TIMPs) Gene Expression

The expression of matrix remodeling enzymes (MMPs), their inhibitors (TIMPs), and cellular fibrinolytic activity genes was assessed over time using RT-PCR (Fig. 4). *Mmp8* (matrix metalloproteinase 8/collagenase-2) and *Mmp9* (matrix metalloproteinase 9/gelatinase B) mRNA expression followed a similar pattern of decline over time, down to nearly 10% of baseline expression (Fig. 4A). In contrast, *Mmp2* (matrix metalloproteinase 2/gelatinase A) and *Mmp13* (matrix metalloproteinase 13/collagenase 3) mRNA expression increased from baseline by nearly 10-folds on day 14, and leveled off thereafter (Fig. 4A). The expression of tissue inhibitor of metalloproteinases 1 (*Timp1*) was only modestly changed from baseline (Fig. 4B), whereas *Timp2* and *Timp3* increased significantly ($p < 0.05$) over time from baseline by nearly 5-folds on day 35 (Fig. 4B).

DISCUSSION

Flexor tendon healing is mediated by cellular contributions from the tendon surface (epitenon), the tendon substance (endotenon), and the synovial sheath in which the tendon glides. These contributions include cell proliferation, migration, and ECM synthesis that contribute to the formation of scar tissue and adhesion [21, 22]. The biological mechanisms of flexor tendon graft repair and adhesion formation remain poorly understood, despite being studied for decades in various animal models including non-human primates [23], canine [24–28], chicken [29–31], rabbits [32–34], and rats [35–37]. To address this, we have previously developed a murine flexor tendon repair model that permits the study of simple repair and segmental reconstruction [16–18, 38, 39]. This murine model provides gene manipulation and transgenic approaches to evaluate the cellular and molecular effects of specific targets on the healing process, as we recently demonstrated [18]. In the murine model, we previously observed that live autografts heal with more extensive scarring and adhesions compared to freeze-dried allografts [16], which suggested that the etiology of adhesions is precipitated intrinsically by the live autograft cells. To test this in the current study, we transplanted live FDL grafts from the reporter mouse *Rosa26^{LacZ/+}* in WT recipients, and used histological X-gal staining to evaluate the intrinsic contribution of the tendon graft to scarring and adhesions. Consistent with our hypothesis, we observed progressive cellular proliferation and migration as evident by outward flux of β -gal-positive graft epitenon cells contributing to the adhesions tissue that filled and obliterated of gliding space. However, there was also evidence of influx of host cells (β -gal-negative) into the gliding space and the graft. Using myeloablated WT mice transplanted with bone marrow from GFP transgenic mice, we previously demonstrated that marrow stem/progenitor cells are mobilized and migrate to the FDL repair site [38]. These observations are in agreement with the literature. For example, Lundborg et al. demonstrated that intrinsic flexor tendon's cells play direct roles in the healing process [40, 41], while Duffy et al. suggested that flexor tendon cells might secrete mitogenic or growth-promoting factors that contribute to the early stages of healing [42]. Others suggested that extrinsic cells contribute to allograft healing. Potenza et al. implanted acellular freeze-dried canine profundus tendon allografts into knee joints of dogs [43], and demonstrated that the allografts were covered by a layer of proliferating fibroblast-like cells that "healed" cuts pre-made in the allografts, and recellularized and remodeled the allografts ends. Clearly, the cells that populated and contributed to the healing of these cuts within the allografts originated from the synovial capsule of the joint, suggesting an extrinsic repair mechanism in intrasynovial environments [43]. Later studies by Manske et al. showed that rabbit flexor tendons with transverse lacerations do in fact possess an intrinsic repair capacity when cultured in vitro in the absence of extrinsic cell sources and without the benefit of nutrition from a circulating blood supply or the influence of synovial fluid [21]. This repair apparently originated from epitenon fibroblasts that proliferated and migrated into the laceration site, forming collagen

fibers that bridged the defect [44]. The literature suggests that flexor tendon repair is accomplished both by extrinsic peripheral cells that likely involve synovial fibroblasts and inflammatory cells and by intrinsic fibroblasts from the tendon itself. The mechanism of adhesion formation is likely precipitated by inflammation. It has been suggested that the presence of inflammatory cells in synovial sheath and epitenon during tendon healing “induces synovial fibroblasts and epitenon cells to increase their production of fibronectin, which provides a scaffold for subsequent adhesion formation,” evidently by secreting growth factors such as TGF- β 1 [36, 45].

The use of the *Rosa26^{LacZ/+}* as a graft donor offers a convenient transgenic model to identify and track the intrinsic contribution of graft cells to the repair as was previously reported for periosteal cell contribution in healing of bone grafts [46]. One limitation of our transgenic approach is that β -gal staining to track the fate of the *Rosa26^{LacZ/+}* graft cells can be confounded by endogenous β -gal activity during cell senescence in the latter stages of repair and fibrosis, which might not distinguish between host and graft cells. β -gal is a known lysosomal enzyme and biomarker of senescence [47]. Thus the observed increase in β -gal activity at later time points might not exclusively identify graft cells, but could also indicate cell senescence. Interestingly, senescence of epithelial cells has been reported to impair repair and exacerbate inflammation effects following epithelial injury, which might contribute to fibrosis leading to chronic obstructive pulmonary disease (COPD) [47]. Thus, the late and persistent increase in senescence-associated β -gal activity in the graft might be also viewed as an indirect marker of aberrant fibrosis in our FDL tendoplasty model. However, we used this simple reporter approach to provide a qualitative assessment of the contribution of graft to the cellular processes involved in healing and scar formation. More direct approaches of assessing cell proliferation and migration, including Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) labeling and immunohistochemistry, will provide a better understanding of these phenomena in future studies.

Growth factors including PDGF, IGF, FGF and TGF- β , as well as other cytokines stimulate the migration and proliferation of fibroblasts and extracellular matrix (ECM) synthesis [48]. These factors also modulate the synthesis and activation of matrix metalloproteinases (MMPs) that act to degrade and turnover the ECM. The balance between ECM synthesis and turnover determines the extent of tissue remodeling into a mature fibrous tissue, which represents the last stage of repair. As such, the outcome of the repair can be variable and can lead to fibrotic scarring. TGF- β has been shown to be critical to the modulation of wound healing and scarring and the effects of its isoforms are quite distinct. For example, the role of TGF- β 1 in scar formation is accentuated by the observation that while fetal cutaneous wound healing and tendon repair are scar-free [49, 50], the in utero administration of TGF- β 1 to fetal rabbit wounds results in fibrotic scarring similar to that observed in adult rabbits [50]. And while there are numerous differences between adult and embryonic wound healing, the temporal expression of the TGF- β isoforms are highly associated with scar formation or the lack thereof, respectively. For example, TGF- β 1 and TGF- β 2 are abundant early on in adult wounds, but they are very low and nearly absent in embryonic scar-less wounds. TGF- β 3, which is highly induced in developing skin and embryonic scar-less wounds, is induced late and at low levels in adult wounds only after TGF- β 1 levels start to fall [51]. These observations motivated the development of experimental anti-TGF- β 1 therapies for flexor tendon adhesions which demonstrated promising early results. For example, neutralizing antibodies to TGF- β 1 reduced flexor tendon adhesions following transection in a rat model [52] and treatment with TGF- β 1 inhibitors such as decorin or mannose-6-phosphate improved the postoperative range of motion in rabbit zone II flexor tendon repair [33].

TGF- β signals by binding to type-II receptor (encoded by *Tgfb2*), which phosphorylates two distinct type-I receptors; ALK-5 receptor (encoded by *Tgfb1* or *Alk-5*) and ALK-1 receptor (encoded by *Acvr1* or *Alk-1*) [53]. In the current study, we observed a persistent upregulation of *Tgfb2* expression, and a bimodal expression profile for *Acvr1* and *Tgfb1*. While the temporal patterns of expression of these type-I receptors appear similar, their signaling pathways and target cells are likely divergent. Signaling through ALK-5 leads to Smad2/3 phosphorylation and while ALK-1 phosphorylates Smad1/5/8 [53]. TGF- β has been shown to signal through both receptors, and the two receptor-kinase phosphorylation pathways are not mutually exclusive. A study investigating TGF- β signaling in EC identified a complex interplay between the two receptors/signaling pathways. For example, it has been shown that ALK-5 plays a role in TGF- β -dependent recruitment of ALK-1 into a TGF- β receptor complex and that the ALK-5 kinase activity is required for optimal ALK-1 activation, while ALK-1 directly antagonizes ALK-5/Smad2,3 signaling [54]. It has also been shown that TGF- β /ALK-5 signaling leads to epithelial cell migration in lung fibrosis [55] and that selective inhibition of ALK-5 signaling blocks profibrotic TGF- β responses in skin fibroblasts [56]. Moreover, we recently demonstrated that Smad3 knockout mice heal flexor tendon lacerations with reduced scarring and improved range of motion compared to WT controls [18]. Collectively, these studies suggest that TGF- β /ALK-5 smad2/3-mediated signaling promotes the fibrotic response and that ALK-1 signaling might antagonize that pathway. However, the exact roles of TGF- β , TGF- β receptors and their Smad-dependent signaling are not completely understood in the context of flexor tendon healing, and their effects on various cellular responses merit further investigation in this system.

Scar tissue is characterized by excessive accumulation of collagen and other ECM components [57]. Collagen, Type I ($[\alpha 1(I)]_2\alpha 2(I)$) and Type III ($[\alpha 1(III)]_3$) play important roles in tendon healing. The early elevated level of expression of all collagen genes (*Col1a1* and *Col3a1*) and their crosslinking enzyme lysyl oxidase (*Lox*) in this experiment, and the subsequent reduction to baseline values by day 35 suggest that the healing process in the murine tendon might be completed over the course of 4–5 weeks making this well characterized model highly feasible for testing interventions with the potential to improve the functional outcomes. These observations are consistent with our previous finding in this model that demonstrated that the normal gliding function and range of motion and tensile strength improve and plateau after 35 or 42 days of healing [16–18]. These observations are also consistent with data in other models of tendon healing, with some key differences in the temporal pattern of expression that likely reflect species differences in healing [35, 58]. *Lox*, which initiates the crosslinking of lysine-derived aldehyde, plays an important role in the maturation of collagen. Studies show that the *Lox* and *Col1a1* promoters may be regulated by TGF- β response elements in rats [59] and mice [60]. Consistent with this, *in vitro* studies have shown that TGF- β 1 causes an increase in *Lox* mRNA expression in murine tail tenocytes [61].

The transition of the fibroblasts into a contractile and migratory α -SMA-expressing myofibroblasts and the upregulation of fibronectin, are thought to be requisites for cell-mediated closure of cutaneous wound. This phenotypic transition has also been observed during anterior cruciate ligament graft remodeling [62] and during the healing of rabbit medial collateral ligament [63]. Interestingly, myofibroblasts are observed at elevated levels in pathologic Dupuytren's tissues [64], suggesting that myofibroblasts are associated with aberrant proliferation, fibrosis and scarring. Furthermore, inflammatory cells and signals have been shown to stimulate synovial and epitenon fibroblasts in rat flexor tendons, mainly through TGF- β 1, to produce of fibronectin, a marker of scarring [36, 45]. In the current study, the observed increases in gene expression of α -SMA and fibronectin suggest that murine flexor tendon heals by scar tissue. Failure to remodel the scar is a precipitating factor in pathologic fibrosis and flexor tendon adhesions.

Matrix MMPs are required for connective tissue remodeling, and have been reported to be upregulated in tendon repair and in tendinopathy [65, 66]. In the current study, we observed that the matrix MMPs (MMP-2 and -13) were progressively upregulated over time, whereas the expression of the inflammatory MMPs (MMP-8 and -9) was highest on day 3 and decreased thereafter. These results are consistent with our previous findings [17]. Expression and activation of MMP-2 has been reported during the healing process of acute supraspinatus tendon tears in rabbits [67]. Similarly, MMP-13 is upregulated in the granulation tissue of equine superficial digital flexor tendinitis [68]. Interestingly, *Mmp8*^{-/-} mice show a significant delay in wound closure with an altered inflammatory response and TGF- β signaling [69]. We have also shown that bone marrow-specific loss of *Mmp9* leads to earlier remodeling of adhesions, without a decrease in the strength of the repair [38]. It is important to note that there are no universally accepted biomarkers for tendon healing or pathology. However, a recent clinical study examined mRNA biomarkers and histological characteristics of repaired rotator cuffs that healed or developed persistent defects, and demonstrated that there was a generalized increase in MMP expression in tendons that failed to heal [70], suggesting that MMP expression might be an important biomarker of pathology.

Our study is not without limitations. A major limitation of this study is that the data is limited to gene expression and lack functional readouts such as MMP activity. MMPs are expressed as pro-MMPs and can be activated by plasmin, an enzyme that in turn is derived from plasminogen via a proteolytic process mediated by plasminogen activator (tPA or uPA). TGF- β 1 has been shown to stimulate the activity of the plasminogen activator inhibitor 1 (PAI-1) in these cells [71] to effectively suppress MMP activity (e.g. MMP-2). Interestingly, TGF- β 3 deficiency has been shown to increase PAI-1 expression and activity in *Tgfb3*^{KO} mouse skin [72], which suggests that TGF- β 1 and TGF- β 3 have opposite effects on PAI-1 expression and activity, and by corollary on MMPs activation. Furthermore, activated MMPs can be regulated by endogenous inhibitors including TIMPs. TGF- β 1 has been reported to modulate the homeostasis between MMPs and MMP inhibitors in cancer cells [73], but its role in regulating MMPs and TIMPs during tendon healing remains unclear, especially that most MMPs have multiple substrates. Thus, the relationship between TGF- β and MMP expression and activity merits further investigation using functional assays that include immunohistochemistry and in vivo imaging of MMP activity in future experiments.

The graft implantation outside of zone II presents another major limitation of our model. Microscopic and histological examination of the mouse hind paw digit and flexor tendon arrangement have recently shown that there is indeed an area where both deep and superficial tendons reside between two pulleys, similar to zone II in the human hand [74]. Because the dimensions of the mouse digit pose insurmountable technical challenges for *in vivo* surgical intervention in those areas, and in the interest of creating a reproducible *in vivo* mouse model of flexor tendoplasty with functional outcomes, we decided not to involve zone II. Though our model is not a true Zone II model, the injury model resulted in impaired joint flexion due to consistent fibrotic scarring and adhesion formation especially around the live autografts [16] similar to that observed in larger animals. In addition, severing the musculotendinous junction to protect the repair integrity also shields the graft from *in vivo* loading, which can confound the natural history of healing. Early (active or passive) mobilization has been shown to be clinically beneficial for functional flexor tendon healing [5, 75]. Preclinical animal studies also demonstrated that passive mobilization of injured canine flexor digitorum profundus tendons increased tensile strength, restored the gliding surfaces of the tendons, and improved excursion properties compared to immobilized controls [76, 77]. However clinical complications due to inadequate repair suture materials and methods or excessive muscle pull typically lead to gap formation that

can delay or inhibit complete healing [78]. Given the size of the mouse model, providing adequate suture support to prevent gapping upon resumption of *in vivo* loading is not feasible, which necessitated severing the myotendinous junction. Given that the mouse is currently the species of choice for the *in vivo* study of various skeletal tissues' disease and repair processes including tendon repair [19, 20], we believe that the advantages of this mouse model outweigh its limitations.

Conclusions

Our study demonstrates that both intrinsic (tendon) and extrinsic cells contributed to the adhesions that obliterate the gliding space. The temporal pattern of expression of ECM proteins and matricellular proteins confirms that murine flexor tendon heals by scar formation, which precipitates adhesions. The biphasic pattern of gene expression of TGF- β proteins suggest that TGF- β 1 and TGF- β 3 are associated with scar formation and remodeling, respectively. MMPs are important for scar remodeling and the prevention of adhesions, and the relationship between TGF- β signaling and MMP expression and activity warrant more detailed mechanistic studies.

Acknowledgments

We would like to thank Drs. Justin Jacobson, Tony Chen, Chao Xie, Matthew Hilton, Alicia Clementi and Sarah Offley for valuable advice and insights during this work. The study was supported by Grants R01AR056696 and P30AR061307 from NIAMS/NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or NIH.

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Figure 1. Intrinsic (tendon) and extrinsic cells contribute to adhesions that obliterate the grafted FDL tendon gliding space

Representative histological sections of Wild type FDL tendons reconstructed with *Rosa26^{LacZ/+}* grafts, stained for β -gal activity using X-Gal as a substrate. Panels A, E, I, M, and Q show the host-graft junction (10 $\times$), while panels B, F, J, N, and R show the graft midsubstance (10 $\times$), and the remaining panels show the region of interest marked by the dotted rectangles at higher magnification (20 $\times$ and 40 $\times$, respectively). (A–D) On day 7, the cellularity of the tendon remains sparse but an outward flux of intrinsic (blue) graft epitenon cells (β -gal-positive) could be observed. At the same time, β -gal-negative cells (extrinsic) could also be observed (shown at higher magnification in D). (E–H) On day 14, graft cellularity (β -gal activity) increased and outward flux of β -gal-positive cells from the graft, contributing to the adhesions that obliterate the gliding space, was more pronounced (shown at higher magnification in H). (I–L) On day 21, β -gal activity in the tendon graft and surrounding adhesions increased. The graft margins were not distinguishable from the surrounding tissues (shown at higher magnification in L). (M–P) Noticeable disorganization of the graft and a loss of the characteristically crimped fibers were observed on day 28. FDL, flexor digitorum longus; β -gal, β -galactosidase.

Figure 2. Temporal Gene expression of TGF- β and its receptors in intercalary *Rosa26^{LacZ/+}* grafts and host FDL tendon junctions over time up to 35 days postsurgery
 Gene expression was quantified using real-time RT-PCR using primers for (A) TGF- β 1 (*Tgfb1*) and TGF- β 3 (*Tgfb3*), and (B) type-II TGF- β receptor (*Tgfb2*), the broadly expressed ALK-5 receptor (*Tgfb1*), and the endothelial cell specific ALK-1 receptor (*Acvr11*). Gene expression was computed relative to the house keeping gene β -actin (*Actb*), and then normalized to baseline (day 3) values. Data plotted on a semi-log scale represent means and error bars represent SEM (n=6 grafted tendons per time point). For each gene, color-matched asterisks indicate significant differences from baseline (p<0.05).

Figure 3. Temporal Gene expression of ECM proteins in intercalary *Rosa26^{LacZ/+}* grafts and host FDL tendon junctions over time up to 35 days postsurgery
 Gene expression was quantified using real-time RT-PCR using primers for (A) collagen types I (*Col1a1*) and III (*Col3a1*) and their crosslinking enzyme lysyl oxidase (*Lox*); and (B) α -SMA (*Acta2*) and Fibronectin (*Fn1*). Gene expression was computed relative to the house keeping gene β -actin (*Actb*), and then normalized to baseline (day 3) values. Data plotted on a semi-log scale represent means and error bars represent SEM (n=6 grafted tendons per time point). For each gene, color-matched asterisks indicate significant differences from baseline ($p<0.05$).

Figure 4. Temporal Gene expression of MMPs and their regulators in intercalary *Rosa26^{LacZ/+}* grafts and host FDL tendon junctions over time up to 35 days postsurgery

Gene expression was quantified using real-time RT-PCR using primers for (A) MMP-2 (*Mmp2*), MMP-8 (*Mmp8*), MMP-9 (*Mmp9*), and MMP-13 (*Mmp13*); and (B) tissue inhibitors of matrix metalloproteinases TIMP-1 (*Timp1*), -2 (*Timp2*), and -3 (*Timp3*). Gene expression was computed relative to the house keeping gene β -actin (*Actb*), and then normalized to baseline (day 3) values. Data plotted on a semi-log scale represent means and error bars represent SEM (n=6 grafted tendons per time point). For each gene, color-matched asterisks indicate significant differences from baseline ($p < 0.05$).

Table 1

Sequences of primers used for Real-time RT-PCR

Gene	Primer Sequence (5' - 3')	
	Forward	Reverse
<i>Tgfb1</i>	AGCAGTCCCCGAACCCCAT	GGGGTCAGCAGCCGGTTACC
<i>Tgfb3</i>	CCACAAGGGGCACCGCTGAA	CCCAGGTCTCCACGGCCATG
<i>Acvr1l</i>	CTCTGCTCGCCTCACCGCAC	CCTGGTGGCCCTGGGCTAGT
<i>Tgfb1</i>	TGGCAGAGCTGTGAGGCCTT	GGGCCCAGAGAAACCCTGGGA
<i>Tgfb2</i>	GCACCAGGGGCCGGTCTATG	TGGGCTTCCATTTCACATCCGACT
<i>Col1a1</i>	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
<i>Col3a1</i>	GCCCACAGCCTTCTACAC	CCAGGGTCACCATTTCCTC
<i>Lox</i>	CGACGACCCCTACAATCCCTACAA	TGCGGAAATCGTAGCAGTACCCT
<i>Acta2</i>	GAGAAGCCCAGCCAGTCG	CTCTTGCTCTGGGCTTCA
<i>Fn1</i>	TTAAGCTCACATGCCAGTGC	TCGTCATAGCACGTTGCTTC
<i>Mmp2</i>	CAAGTTCCCCGGCGATGTC	TTCTGGTCAAGGTCACCTGTC
<i>Mmp8</i>	TCTTCCTCCACACACAGCTTG	CTGCAACCATCGTGGCATTTC
<i>Mmp9</i>	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
<i>Mmp13</i>	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCAGTGTAGACT
<i>Timp1</i>	CCATGATGGCCCCCTTTGCATCT	ACTGGGGTGTAGGCGTACCGG
<i>Timp2</i>	CCCGCAACAGGCGTTTTGCA	CTGCTGAAGAGGGGGCCGTG
<i>Timp3</i>	CCGCTACCACCTGGGTTGCA	TCTGCCGGATGCAGGCGTAG
<i>Actb</i>	CTTCTTGGGTATGGAATCCTGTGGC	AAACGCAGCTCAGTAACAGTCCGC